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⑤④ Analogous interferon polypeptides, process for their preparation and pharmaceutical compositions containing them.

⑤⑦ Analogues of human Interferon  $\alpha_2$  in which an amino acid is present in at least one position selected from Interferon  $\alpha_2$  positions 12-16, 21, 27-32, 37, 43a, 44, 59, 61, 64, 67, 98-115, 137-138 and 148 which differs from the amino acid in the corresponding position of Interferon  $\alpha_2$ , possess good antiviral activity and may additionally be used as biochemical tools. The interferon analogues are prepared by culturing a microorganism, the microorganism having been transformed with a replicable plasmidic expression vehicle comprising genetic material coding for the said interferon analogue.

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## TITLE MODIFIED

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POLYPEPTIDES

The present invention relates to polypeptides, particularly interferon analogues, such as analogues of human interferon  $\alpha_2$ , processes for their preparation and pharmaceutical compositions containing them as well as to the genetic modification of microorganisms to express the said analogues, the microorganisms thereby obtained, the genetic material employed in the modification and vectors therefor. The human interferon  $\alpha_2$  (hereinafter also referred to as IFN- $\alpha_2$ ) analogues of the present invention possess interesting physiological activity.

Natural interferons are known compounds which are produced by a variety of living cells both in vivo and in vitro in tissue cultures in response to certain inducers, such as viral infection, immune stimulation and a range of chemical inducers. They are defined as proteins able to inhibit the replication of a variety of RNA and DNA viruses through cellular metabolic processes involving the synthesis of new RNA and protein (Committee on Interferon Nomenclature, 1980). Three main classes of natural interferons have been distinguished to date and are currently named alpha (IFN- $\alpha$ ), beta (IFN- $\beta$ ) and gamma (IFN- $\gamma$ ) on the basis of antigenic types. Interferons, for example, IFN-alpha possess antiviral, antiproliferative and immunomodulatory activity.

Information on the biological properties of interferons has come predominantly from studies of material produced by cell culture techniques. Recent results using recombinant DNA techniques have shown that human leucocyte interferon (Hu-IFN-alpha) is a family of at least 8 different molecules (Goeddel et al, Nature, 1981, 290, pages 20-26). Furthermore, Allen and Fantes

(Nature, 1980, 287 pages 408-411) have demonstrated that microheterogeneities in interferon preparations from Namalwa cells can be ascribed to the presence of at least five homologous proteins with different amino acid sequences and hence to different structural genes. Despite these advances, very little is known about the distinct physiological properties of individual sub-species of Hu-IFN-alpha.

As stated above human leucocyte interferon is a family of a number of different molecules such as IFN-alpha<sub>1</sub> and IFN-alpha<sub>2</sub>, the present invention being concerned inter alia with analogues of IFN-alpha<sub>2</sub>.

The preparation of certain truncated analogues of IFN-alpha<sub>2</sub> has been described in the literature. Thus it is stated in Weck, P. K. et al. J. Cell. Biochem. Suppl. 6:104, 1982 that studies with hybrid leukocyte interferons indicate that both the N- and the C-terminal portions of the leukocyte interferons are involved in receptor binding. These studies involved certain hybrids and indicated that the C-terminal 15 amino acids clearly have significant effects on the activity of these interferons. Nevertheless a number of analogues of interferon alpha<sub>2</sub> have been prepared having a truncated C-terminus and for example, IFN-alpha<sub>2</sub> (1-160), IFN-alpha<sub>2</sub> (1-155) and IFN-alpha<sub>2</sub> (1-152) have all been reported to retain their antiviral activity, but show no adequate separation of antiviral from antiproliferative or NK-cell stimulating activities relative to IFN-alpha<sub>2</sub>.

IFN-alpha<sub>2</sub> (4-160) and IFN-alpha<sub>2</sub> (4-165) have also been disclosed, but these truncated analogues also showed no significant separation of antiviral from antiproliferative or NK-cell stimulating activities relative to IFN-alpha<sub>2</sub>. We have found, however, that IFN-alpha<sub>2</sub> (4-155) showed a consistent ten-fold increase

over IFN- $\alpha_2$  in the number of antiviral units required to produce a 50% inhibition of Daudi cell proliferation.

The literature also contains a number of references to the production of terminal hybrids of IFN- $\alpha_2$  with IFN- $\alpha_1$  or with IFN- $\alpha$  I. It will be understood that references herein to a terminal hybrid mean a hybrid of two interferon molecules in which the hybrid consists of two sections, the N-terminal section of the hybrid comprising the amino acids in the sequence of one interferon and the C-terminal section comprising the amino acids in the sequence of the other interferon. Thus IFN- $\alpha_1$  (1-92)/ $\alpha_2$  (92-165), IFN- $\alpha_1$  (1-62)/ $\alpha_2$  (62-165), IFN- $\alpha_2$  (1-91)/ $\alpha_1$  (93-166) and IFN- $\alpha_2$  (1-61)/ $\alpha_1$  (63-166) have all been described as possessing antiviral activity in Weck, P. K. et al (1981) "Antiviral activities of hybrids of two major human leukocyte interferons", Nucleic Acids Research 9, 6153-6166. IFN- $\alpha_2$  (1-149)/ $\alpha$ -I (151-166), IFN- $\alpha$ -I (1-150)/ $\alpha_2$  (150-165), IFN- $\alpha_2$  (1-149)/ $\alpha_1$  (151-166) and IFN- $\alpha_1$  (1-150)/ $\alpha_2$  (150-165) have all been described as possessing antiviral activity in Franke, A. E. et al (1982). "Carboxyterminal region of hybrid leukocyte interferons affects antiviral specificity". DNA, 1, 223-230.

One internal  $\alpha_1/\alpha_2$  hybrid (in which an IFN- $\alpha_1$  segment is flanked by segments of interferon  $\alpha_2$ ) has been disclosed as possessing antiviral activity and this analogue is [Thr<sup>68</sup>, Asp<sup>79</sup>, Cys<sup>85</sup>]IFN- $\alpha_2$ . This analogue is disclosed in Rehberg, E. et al (1982) "Specific molecular activities of recombinant and hybrid leukocyte interferons" Journal of Biological Chemistry, 257, 11497-11502.

The present invention is based on the discovery that novel polypeptides possessing at least certain of the properties of natural interferons, but in particular good antiviral activity, may be prepared by effecting one or more amino acid or sequence changes as hereinafter defined to the structure of IFN- $\alpha_2$  or its known analogues. Whilst the amino acid sequence for IFN- $\alpha_2$  is known, it is not known, and it cannot be accurately predicted, what changes to the IFN- $\alpha_2$  polypeptide may be made without losing antiviral activity. It is known that apparently small changes to polypeptide molecules made in one region of the polypeptide may destroy all biological activity whereas an improvement in biological activity may be obtained by an equally small change in a different region of the polypeptide.

The polypeptides of the present invention may also be used as biochemical tools, for example, after immobilization on an appropriate support they may be used for the enrichment and purification of interferon receptors or they may be useful in discriminating between families of monoclonal antibodies raised against native interferons.

It is to be understood that in this specification the abbreviations used for amino acids are standard abbreviations used in the peptide art (see Pure and Applied Chemistry, 1974, 40, 317-331, and Neuropeptides, 1981, 1, 231-235). Thus for example the following amino acids are abbreviated as indicated:-

- 5 -

	Alanine	Ala	Leucine	Leu
	Arginine	Arg	Lysine	Lys
	Asparagine	Asn	Methionine	Met
	Aspartic acid	Asp	Phenylalanine	Phe
5	Glutamic acid	Glu	Proline	Pro
	Glutamine	Gln	Serine	Ser
	Glycine	Gly	Threonine	Thr
	Histidine	His	Tryptophan	Trp
	Isoleucine	Ile	Tyrosine	Tyr
10	Cysteine	Cys	Valine	Val

The configuration of a particular amino acid, other than glycine, will be understood to be the natural L-configuration.

According to one feature of the present  
 15 invention we thus provide a polypeptide of the formula

I:-

X<sup>1</sup>-X<sup>2</sup>-X<sup>3</sup>-X<sup>4</sup>-X<sup>5</sup>-X<sup>6</sup>-X<sup>7</sup>-X<sup>8</sup>-X<sup>9</sup>-X<sup>10</sup>-X<sup>11</sup>-X<sup>12</sup>-X<sup>13</sup>

[X<sup>14</sup>-X<sup>15</sup>-X<sup>16</sup>-Leu-Leu-Ala-Gln-X<sup>21</sup>-X<sup>22</sup>-X<sup>23</sup>-Ile-Ser-X<sup>26</sup>

20

[X<sup>27</sup>-X<sup>28</sup>-X<sup>29</sup>-X<sup>30</sup>-X<sup>31</sup>-X<sup>32</sup>-Arg-X<sup>34</sup>-Asp-Phe-X<sup>37</sup>-X<sup>38</sup>-Pro

[Gln-Glu-Glu-Phe-X<sup>43a</sup>-X<sup>44</sup>-Asn-Gln-Phe-Gln-Lys-X<sup>50</sup>-X<sup>51</sup>

25

[X<sup>52</sup>-Ile-X<sup>54</sup>-Val-Leu-His-Glu-X<sup>59</sup>-Ile-X<sup>61</sup>-Gln-X<sup>63</sup>

[X<sup>64</sup>-Asn-Leu-X<sup>67</sup>-X<sup>68</sup>-Thr-X<sup>70</sup>-Asp-Ser-Ser-Ala-Ala-Trp

[X<sup>77</sup>-X<sup>78</sup>-X<sup>79</sup>-Leu-Leu-X<sup>82</sup>-Lys-Phe-X<sup>85</sup>-Thr-Glu-Leu-Tyr

30

[Gln-Gln-Leu-Asn-X<sup>94</sup>-Leu-Glu-Ala-X<sup>98</sup>-X<sup>99</sup>-X<sup>100</sup>-X<sup>101</sup>

[X<sup>102</sup>-X<sup>103</sup>-X<sup>104</sup>-X<sup>105</sup>-X<sup>106</sup>-X<sup>107</sup>-X<sup>108</sup>-X<sup>109</sup>-X<sup>110</sup>-X<sup>111</sup>

35

[X<sup>112</sup>-X<sup>113</sup>-X<sup>114</sup>-X<sup>115</sup>-Ile-Leu-Ala-Val-X<sup>120</sup>-Lys-Tyr-Phe

[X<sup>124</sup>-Arg-Ile-Thr-Leu-Tyr-Leu-X<sup>131</sup>-Glu-Lys-Lys-Tyr

[Ser-X<sup>137</sup>-X<sup>138</sup>-Ala-Trp-Glu-Val-Val-Arg-Ala-Glu

25

[Ile-X<sup>148</sup>-Arg-Ser-X<sup>151</sup>-Ser-X<sup>153</sup>-Ser-X<sup>155</sup>-X<sup>156</sup>-X<sup>157</sup>

[X<sup>158</sup>-X<sup>159</sup>-X<sup>160</sup>-X<sup>161</sup>-X<sup>162</sup>-X<sup>163</sup>-X<sup>164</sup>-X<sup>165</sup>.

30

in which X<sup>1</sup>, X<sup>2</sup>, X<sup>3</sup>, X<sup>4</sup>, X<sup>5</sup>, X<sup>6</sup>, X<sup>7</sup>, X<sup>8</sup>, X<sup>9</sup>, X<sup>10</sup>, X<sup>11</sup>,  
 X<sup>155</sup>, X<sup>156</sup>, X<sup>157</sup>, X<sup>158</sup>, X<sup>159</sup>, X<sup>160</sup>, X<sup>161</sup>, X<sup>162</sup>, X<sup>163</sup>,  
 X<sup>164</sup> and X<sup>165</sup> may each represent a naturally occurring

alpha-amino acid and X<sup>12</sup> represents Arg or Ala, X<sup>13</sup>  
 represents Arg or Ala, X<sup>14</sup> represents Thr or Ala, X<sup>15</sup>

35

represents Leu or Ala, X<sup>16</sup> represents Met, Leu or Ile,  
 X<sup>21</sup> represents Met or Leu, X<sup>22</sup> represents Ser, Arg or

Gly and X<sup>23</sup> represents Arg or Lys, X<sup>26</sup> represents Leu or  
 Pro, X<sup>27</sup> represents Ser or Phe, X<sup>28</sup> represents Ser or  
 Ala, X<sup>29</sup> represents Ser or Cys, X<sup>30</sup> represents Leu or  
 Ile X<sup>31</sup> represents Met or Lys, X<sup>32</sup> represents Ala, Asn,  
 5 Asp or Glu, X<sup>34</sup> represents His or Pro, X<sup>37</sup> represents  
 Gly, or Ala, X<sup>38</sup> represents Phe or Leu, X<sup>43a</sup> represents  
 a single bond or Asp, X<sup>44</sup> represents Gly or Ala, X<sup>50</sup>  
 represents Ala or Thr, X<sup>51</sup> represents Glu Pro or Glu  
 X<sup>52</sup> represents Ala or Thr, and X<sup>54</sup> represents Ser or  
 10 Pro, X<sup>59</sup> represents Leu or Met, X<sup>61</sup> represents Glu or  
 Ala, X<sup>63</sup> represents Ile or Thr, X<sup>64</sup> represents Phe or  
 Ala, X<sup>67</sup> represents Phe or Ala, X<sup>68</sup> represents Thr or  
 Ser, X<sup>70</sup> represents Lys or Glu, X<sup>77</sup> represents Asp or  
 Glu, X<sup>78</sup> represents Glu or Gln, X<sup>79</sup> represents Asp, Thr  
 15 or Ser, X<sup>82</sup> represents Asp or Glu X<sup>85</sup> represents Cys,  
 Tyr or Ser, X<sup>94</sup> represents Asp or Asn, X<sup>98</sup> represents  
 Cys or Leu, X<sup>99</sup> represents Val or Thr, X<sup>100</sup> represents  
 Met, Ile or Asn, either X<sup>101</sup> represents Gln, Tyr or  
 Phe, X<sup>102</sup> represents Glu, Gly, Ser or Ala, X<sup>103</sup>  
 20 represents Glu, Val or Lys, X<sup>104</sup> represents Arg, Gly,  
 Thr, Leu or Ala, X<sup>105</sup> represents Val, Asp, Met or Thr,  
 X<sup>106</sup> represents Gly, Thr, Glu, Leu or Asn, X<sup>107</sup>  
 represents Glu, Asn or Tyr, X<sup>108</sup> represents Thr or Val,  
 X<sup>109</sup> represents Pro or Gln, X<sup>110</sup> represents Leu or Arg  
 25 and X<sup>111</sup> represents Met, Lys or Leu,  
 or -X<sup>101</sup>-X<sup>102</sup>-X<sup>103</sup>-X<sup>104</sup>-X<sup>105</sup>-X<sup>106</sup>-X<sup>107</sup>-  
 X<sup>108</sup>-X<sup>109</sup>-X<sup>110</sup>-X<sup>111</sup>- represents Met, Lys or Leu,  
 -X<sup>112</sup> represents Asn, Lys or Ala, X<sup>113</sup> represents Ala,  
 Glu or Ile, X<sup>114</sup> represents Asp or His, X<sup>115</sup> represents  
 30 Ser or Phe, X<sup>120</sup> represents Lys or Arg, X<sup>124</sup> represents  
 Arg or Gln, X<sup>131</sup> represents Thr or Lys, X<sup>137</sup> represents  
 Pro or Ala, X<sup>138</sup> represents Cys or Ser, X<sup>148</sup> represents  
 Met or Leu, X<sup>151</sup> represents Leu or Phe, X<sup>153</sup> represents  
 Leu or Phe, the amino acids being selected such that an

amino acid is present in at least one position selected from  $\alpha_2$  positions 12-16, 21, 28, 29, 30, 32, 37, 44, 61, 64, 67, 98-115, 137, 138 and 148 which differs from the amino acid in the corresponding position of IFN-

5  $\alpha_2$ , IFN- $\alpha_1$  and IFN- $\alpha$  and/or selected such that an amino acid is absent from at least one position selected from  $\alpha_2$  positions 101-110;

or the amino acids being selected such that  
10 an amino acid is present in at least one position selected from  $\alpha_2$  positions 27, 31, 59, 151, 160 and 163 which is the same as the amino acid in the corresponding position of IFN- $\alpha_1$ , the amino acids at  $\alpha_2$  positions other than positions 1-11, 27, 31, 43a, 59, 100, 102-104, 106, 112, 113, 151 and 156-165 being  
15 the same as the amino acids in the corresponding positions of IFN- $\alpha_2$ ) or a  $N_{1-11}$  and/or  $C_{1-11}$  truncated analogue thereof and the  $N_{3-11}$  truncated analogues of IFN- $\alpha_2$ , which may if desired be  $C_{1-11}$  truncated, with the proviso that a  $N_3$ -truncated analogue  
20 of IFN- $\alpha_2$  is also  $C_{7-11}$  truncated.

It will be appreciated that the amino acids at positions 1 to 11 and 155 to 165 are selected so as not to destroy the overall conformation of the molecule. Thus it is preferred that a variety of different amino  
25 acids should precede the  $N^{12}$  amino acid residue and follow the  $N^{154}$  amino acid residue. Polypeptides in which all amino acid residues preceding  $N^{12}$  and/or following  $N^{154}$  are the same, especially where that amino acid is cysteine should be avoided. Similarly the  
30 presence of repeated (for example more than six) hydrophobic or repeated (for example more than six) charged amino acid residues in sequence in either the  $N_{1-11}$  or  $C_{1-11}$  portion of the polypeptide is preferably avoided.

In the polypeptide of formula I preferably  
x<sup>1</sup> represents Cys, x<sup>2</sup> represents Asp or Trp, x<sup>3</sup>  
represents Leu or Cys, x<sup>4</sup> represents Pro or Gln, x<sup>5</sup>  
represents Glu, Gln or Asp, x<sup>6</sup> represents Thr or Pro, x<sup>7</sup>  
5 represents His or Tyr, x<sup>8</sup> represents Ser, x<sup>9</sup> represents  
Leu, x<sup>10</sup> represents Asp, Gly or Ala, x<sup>11</sup> represents Asn,  
Ser or Ala, x<sup>155</sup> represents Thr, x<sup>156</sup> represents Asn,  
x<sup>157</sup> represents Leu, x<sup>158</sup> represents Gln, x<sup>159</sup>  
represents Glu or Lys, x<sup>160</sup> represents Arg, Ser or Ile,  
10 x<sup>161</sup> represents Leu, x<sup>162</sup> represents Arg, x<sup>163</sup>  
represents Arg or Ser, x<sup>164</sup> represents Lys and x<sup>165</sup>  
represents Glu or Asp.

It will be understood that references herein to an N<sub>1-11</sub>  
truncated analogue mean an analogue of the polypeptide  
15 of formula I which is shortened by the absence from the  
N-terminal end of the polypeptide of any of the first 1-  
11 amino acids without a sequence change. Preferably  
such references indicate an analogue of the polypeptide  
of formula I in which the first, first two or first  
20 three, amino acids are absent from the N-terminal end of  
the polypeptide of formula I. Similarly references  
herein to a C<sub>1-11</sub> truncated analogue mean an analogue of  
the polypeptide in which the C-terminal end of the  
polypeptide is shortened by the absence of any of the  
25 last 1-11 C-terminal amino acids without a sequence  
change being effected. Preferably such references  
indicate an analogue in which the last, last two, last  
three, last four, last five, last six, last seven, last  
eight, last nine or last ten amino acids are absent from  
30 the C-terminal end of the polypeptide.

It will be appreciated that the  
polypeptide of formula I may carry a methionine at its  
N-terminus. Moreover the polypeptide of formula I may  
carry additional amino acid residues at its N-terminus  
35 which in turn may be preceded by methionine. Preferably  
the polypeptide of formula I will carry no more than 17

additional amino acid residues at its N-terminus.

It will also be appreciated that the polypeptides of the present invention may if desired be radiolabelled. The radiolabel may for example be present at the sulphur of a methionine or it may be present as an iodine substituent on tyrosine.

The polypeptides of the present invention thus encompass certain N<sub>3-11</sub>, truncated analogues of IFN-alpha<sub>2</sub> per se such as IFN-alpha<sub>2</sub> (10-165) and IFN-alpha<sub>2</sub> (12-165) as well as such analogues which are additionally C<sub>1-11</sub> preferably C<sub>1-10</sub> C-truncated such as IFN-alpha<sub>2</sub> (4-155). Indeed in our hands we have found that IFN-alpha<sub>2</sub> (4-155) has a particularly good relative antiviral potency in terms of the ratio of antiviral activity to antiproliferative activity and thus IFN-alpha<sub>2</sub> (4-155) is a preferred polypeptide of the invention.

In one particular embodiment, the present invention relates to polypeptides of formula I other than truncated analogues.

The present invention also encompasses for example polypeptides of the formula II:

Cys-Asp-Leu-Pro-x<sup>5a</sup>-Thr-His-Ser-Leu-x<sup>10a</sup>-x<sup>11a</sup>-Arg-Arg-  
 Thr-Leu-Met-Leu-Leu-Ala-Gln-Met-Arg-Lys-Ile-Ser-Leu-  
 x<sup>27a</sup>-Ser-Cys-Leu-x<sup>31a</sup>-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-  
 Gln-Glu-Glu-Phe-x<sup>43a</sup>-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-  
 Tyr-Ile-Pro-Val-Leu-His-Glu-x<sup>59a</sup>-Ile-Gln-Gln-Ile-Phe-  
 Asn-Leu-Phe-Ser-Thr-Lys-Asp-Ser-Ser-Ala-Ala-Trp-Asp-  
 Glu-Thr-Leu-Leu-Asp-Lys-Phe-Tyr-Thr-Glu-Leu-Tyr-Gln-

-Gln-Leu-Asn-Asp-Leu-Glu-Ala-Cys-Val-X<sup>100a</sup>-Gln-  
 X<sup>102a</sup>-X<sup>103a</sup>-X<sup>104a</sup>-Val-X<sup>106a</sup>-Glu- Thr-Pro-Leu-Met-  
 5 X<sup>112a</sup>-X<sup>113a</sup>-Asp-Ser-Ile-Leu-Ala-Val-Arg-Lys-Tyr-Phe-  
 Gln-Arg-Ile-Thr-Leu-Tyr-Leu-Lys-Glu-Lys-Lys-Tyr-Ser-  
 Pro-Cys-Ala-Trp-Glu-Val-Val-Arg-Ala-Gln-Ile-Met-Arg-  
 10 Ser-X<sup>151a</sup>-Ser-Leu-Ser-Thr-Asn-Leu-Gln-Glu-X<sup>160a</sup>-  
 Leu-Arg-X<sup>163a</sup>-Lys-Glu (in which X<sup>5a</sup> represents Glu or  
 15 Gln, X<sup>10</sup> represents Asp or Gly, X<sup>11</sup> represents Asn or  
 Ser, X<sup>27a</sup> represents Phe or Ser, X<sup>31a</sup> represents Lys or  
 Met; X<sup>43a</sup> represents Asp or a single bond, X<sup>59a</sup>  
 represents Met or Leu, X<sup>100a</sup> represents Met or Ile,  
 X<sup>102a</sup> represents Glu or Gly, X<sup>103a</sup> represents Glu or  
 20 Val, X<sup>104a</sup> represents Arg or Gly, X<sup>106a</sup> represents Gly  
 or Thr, X<sup>112a</sup> represents Asn or Lys, X<sup>113a</sup> represents  
 Ala or Glu, X<sup>151a</sup> represents Leu or Phe, X<sup>160a</sup>  
 represents Arg or Ser and X<sup>163a</sup> represents Arg or Ser  
 with the proviso that the amino acids are selected such  
 25 that at least one of X<sup>27a</sup>, X<sup>31a</sup>, X<sup>59a</sup>, X<sup>151a</sup>, X<sup>160a</sup> and  
 X<sup>163a</sup> represents the same amino acid as that in the  
 corresponding position of IFN-alpha<sub>1</sub>) or a N<sub>1-11</sub> or C<sub>1-11</sub>  
 truncated analogue thereof.  
 Preferred polypeptides of formula II by virtue of  
 30 their improved relative antiviral potency in terms of  
 the ratio of antiviral activity to antiproliferative  
 activity are compounds of formula II in which X<sup>43a</sup>  
 represents Asp, X<sup>100a</sup> represents Ile, X<sup>102a</sup> represents  
 Gly, X<sup>103a</sup> represents Val, X<sup>104a</sup>

represents Gly,  $x^{106a}$  represents Thr,  $x^{112a}$  represents Lys and  $x^{113a}$  represents Glu with the proviso that at least one of  $x^{5a}$ ,  $x^{10a}$ ,  $x^{11a}$ ,  $x^{151a}$ ,  $x^{160a}$  and  $x^{163a}$  represents an amino acid which differs from the amino acid in the corresponding position of IFN- $\alpha_2$ .

An especially preferred compound of formula II by virtue of its good ratio of antiviral to antiproliferative activity is endo-Asp<sup>43a</sup>-[Leu<sup>151</sup>, Arg<sup>160</sup>, 163]IFN- $\alpha_2$ .

The polypeptides of the present invention relate in particular to analogues of IFN- $\alpha_2$  of formula I in which an amino acid is present in at least one position selected from  $\alpha_2$  positions 12-16, 21, 28, 29, 30, 32, 37, 44, 61, 64, 67, 98-115, 137, 138 and 148 which differs from the amino acid in the corresponding position of IFN- $\alpha_2$ , IFN- $\alpha_1$  and IFN- $\alpha$ .

In a further embodiment of the present invention there are provided compounds of formula I in which  $x^1$  represents Cys,  $x^2$  represents Asp or Trp,  $x^3$  represents Leu or Cys,  $x^4$  represents Pro or Gln,  $x^5$  represents Gln or Asp,  $x^6$  represents Thr or Pro,  $x^7$  represents His or Tyr,  $x^8$  represents Ser,  $x^9$  represents Leu,  $x^{10}$  represents Gly or Ala,  $x^{11}$  represents Ser or Ala,  $x^{16}$  represents Leu or Met,  $x^{22}$  represents Arg,  $x^{23}$  represents Lys,  $x^{26}$  represents Leu,  $x^{27}$  represents Phe,  $x^{31}$  represents Lys,  $x^{34}$  represents His,  $x^{38}$  represents Phe,  $x^{43a}$  represents a single bond,  $x^{50}$  represents Ala,  $x^{51}$  represents Glu,  $x^{52}$  represents Thr,  $x^{54}$  represents Pro,  $x^{63}$  represents Ile,  $x^{68}$  represents Ser,  $x^{70}$  represents Lys,  $x^{77}$  represents Asp,  $x^{78}$  represents Glu,  $x^{79}$  represents Thr,  $x^{82}$  represents Asp,  $x^{85}$  represents Tyr,  $x^{94}$  represents Asp,  $x^{105}$  represents Val, Asp or Thr,  $x^{106}$  represents Thr, Gly, Leu or Asn,  $x^{120}$  represents Arg,  $x^{124}$  represents Gln,  $x^{131}$  represents

5 Lys, x<sup>151</sup> represents Phe, x<sup>153</sup> represents Leu, x<sup>156</sup> represents Asn, x<sup>157</sup> represents Leu, x<sup>158</sup> represents Gln, x<sup>159</sup> represents Glu, x<sup>160</sup> represents Ser, x<sup>161</sup> represents Leu, x<sup>162</sup> represents Arg, x<sup>163</sup> represents Ser, x<sup>164</sup> represents Lys and x<sup>165</sup> represents Glu) and the N<sub>1-11</sub> and C<sub>1-11</sub> truncated analogues thereof. Where such truncated analogues are obtained they are advantageously the N<sub>1-3</sub> and/or C<sub>1-10</sub> truncated analogues.

10 Generally it is preferred that the compounds of formula I of the present invention contain a Cys<sup>29</sup>, Cys<sup>138</sup> disulphide bridge as in natural IFN-α<sub>2</sub>. We have found that [Ser<sup>29</sup>,Ser<sup>138</sup>]-IFN-α<sub>2</sub>, however, not only possesses antiviral activity, but possesses a relative antiviral potency in terms of the ratio between  
15 antiviral and antiproliferative activity (AV/AP) which is substantially greater than that reported for Hu-IFN-α<sub>2</sub>. This is a particularly surprising finding in view of the belief in the literature that the Cys<sup>29</sup>, Cys<sup>138</sup> disulphide bridge is essential for  
20 activity.

Compounds of the formula:-

Cys x<sup>2</sup>x<sup>3</sup>x<sup>4</sup>x<sup>5</sup>x<sup>6</sup>x<sup>7</sup>-Ser-Leu-x<sup>10c</sup>-Ser-Arg-Arg-Thr-Leu-Met-

[Leu-Leu-Ala-Gln-Met-Arg-Lys-Ile-Ser-Leu-Phe-Ser-Cys]

25 [Leu-Lys-Asp-Arg-His-Asp-Phe-x<sup>37</sup>-Phe-Pro-Gln-Glu-Glu]

[Phe-x<sup>44</sup>-Asn-Gln-Phe-Gln-Phe-Gln-Lys-Ala-Glu-Thr]

30 [Ile-Pro-Val-Leu-His-Glu-x<sup>59</sup>-Ile-Gln-Gln-Ile-Phe-Asn]

[Leu-Phe-Ser-Thr- Lys-Asp-Ser-Ser-Ala-Ala-Trp-Asp-Glu]

[Thr-Leu-Leu-Asp-Lys-Phe-Tyr-Thr-Glu-Leu-Tyr-Gln-Gln]

35 [Leu-Asn- Asp-Leu-Glu-Ala-x<sup>98c</sup>-x<sup>99c</sup>-x<sup>100c</sup>-x<sup>101c</sup>-

-X<sup>102c</sup>-X<sup>103c</sup>-X<sup>104c</sup>-X<sup>105c</sup>-X<sup>106c</sup>-X<sup>107c</sup>-X<sup>108</sup>-X<sup>109</sup>-X<sup>110</sup>  
 [X<sup>111c</sup>-X<sup>112c</sup>-X<sup>113c</sup>-X<sup>114</sup>-Ser-Ile-Leu-Ala-Val-Arg-Lys]  
 5 [Tyr-Phe -Gln-Arg-Ile-Thr-Leu-Tyr-Leu-Lys-Glu-Lys-Lys]  
 [Tyr-Ser-Pro-Cys-Ala-Trp-Glu-Val-Val-Arg-Ala-Glu-Ile]  
 [Met- Arg-Ser-Phe-Ser-Leu-Ser-Thr-Asn-Leu-Gln-Glu-Ser]  
 10 [Leu-Arg-Ser-Lys-Glu  
 [(wherein X<sup>2</sup> represents Asp or Trp, X<sup>3</sup>  
 represents Leu or Cys, X<sup>4</sup> represents Pro or Gln, X<sup>5</sup>  
 15 represents Gln or Asp, X<sup>6</sup> represents Thr or Pro, X<sup>7</sup>  
 represents His or Tyr, X<sup>10c</sup> represents Gly or Ala,  
 X<sup>37</sup> represents Gly or Ala, X<sup>44</sup> represents Gly or Ala, X<sup>59</sup>  
 represents Met or Leu, X<sup>98c</sup> represents Cys or Leu, X<sup>99c</sup>  
 represents Val or Thr, X<sup>100c</sup> represents Ile, Met or Asn,  
 20 X<sup>101c</sup> represents Gln or Tyr, X<sup>102c</sup> represents Gly, Ala or  
 Ser, X<sup>103c</sup> represents Val, X<sup>104c</sup> represents Gly, Ala or  
 Thr, X<sup>105c</sup> represents Val or Asp, X<sup>106c</sup> represents Thr or  
 Leu, X<sup>107c</sup> represents Glu or Asn, X<sup>108</sup> represents Thr or  
 Val, X<sup>109</sup> represents Pro or Gln, X<sup>110</sup> represents Leu or  
 25 Arg, X<sup>111c</sup> represents Met or Lys, X<sup>112c</sup> represents Lys or  
 Ala, X<sup>113c</sup> represents Glu or Ile and X<sup>114</sup> represents Asp  
 or His) and corresponding polypeptides in which Cys at  
 positions 29 and 138 is replaced by Ser, the amino acids  
 being selected such that at least in one of positions  
 30 2-7,10,37,44,59 and 98-114 an amino acid is present which  
 differs from the amino acid in the corresponding  
 position of IFN-alpha<sub>2</sub>] and the N<sub>1-11</sub> (preferably N<sub>1-3</sub>)  
 and C<sub>1-11</sub> (preferably C<sub>1-10</sub>) truncated analogues thereof  
 are preferred because of their good relative antiviral  
 35 potency in terms of the ratio of antiviral to  
 antiproliferative activity.

The following features either alone or in  
 combination are preferred;

- 1) the presence of gamma 98-114, especially gamma  
 101-114, in the Hu-IFN-alpha<sub>2</sub> polypeptide at positions

98-114, especially 101-114 respectively;

2) the presence of alanine in at least one of positions 10,37,44,102 and 104 in the Hu-IFN-alpha<sub>2</sub> polypeptide;

3) the presence of serine at both positions 29 and position 138 of the Hu-IFN-alpha<sub>2</sub> polypeptide;

5 4) the presence of leucine at position 59 of the Hu-IFN-alpha<sub>2</sub> polypeptide.

When any one of these features is taken either singly or in combination, with the other general or particular features of the polypeptides of the present invention, preferred sub-groups of polypeptides are obtained.

Especially preferred polypeptides by virtue of their good relative antiviral potency include:

15 [gamma (98-114)<sup>98-114</sup>]IFN-alpha<sub>2</sub>,  
[Met<sup>100</sup>, gamma(101-114)<sup>101-114</sup>]IFN-alpha<sub>2</sub>,  
[gamma (2-7), gamma (98-114)]IFN-alpha<sub>2</sub>,  
[Leu<sup>59</sup>]IFN-alpha<sub>2</sub>,  
[Ala 10,37,44,102,104]IFN-alpha<sub>2</sub> and  
[Ser<sup>29,138</sup>]IFN-alpha<sub>2</sub>.

20 In the drawings accompanying this specification Figure 1 illustrates the nucleotide sequence of a Hu IFN-alpha<sub>2</sub> gene and the corresponding amino acid sequence; Figure 2 is a diagrammatic representation of the structure of the plasmid pSTPl.

25 The polypeptides of the present invention are conveniently prepared by genetic engineering techniques for example such as described in European Patent Publication No. 0062971A2, the disclosure of which is incorporated herein by way of reference.

30 We thus provide according to a further feature of the present invention a process for producing a polypeptide of formula I as hereinbefore defined which process comprises culturing a microorganism, the microorganism having been transformed with a replicable  
35 plasmidic expression vehicle comprising genetic material coding for the said polypeptide of formula I, whereby to effect expression of the said polypeptide and recovering the said polypeptide thereby expressed.

The above-mentioned process may be effected by the use of any appropriate microorganism such as a gram negative E. coli or an autotrophic bacterium (e.g. one capable of using as its sole or principal carbon source inorganic carbon e.g. carbon dioxide, or a bicarbonate), or a methylotrophic bacterium (e.g. one capable of using as its sole or principal carbon source methane or 'methyl' carbon, for example from methanol). However we do not exclude the use of any of the microorganisms referred to in European Patent Publication No. 0062971A2.

Both anaerobic and aerobic varieties of microorganisms are found to be interest in the process of the present invention, as well as both facultative and obligate organisms, and the selection of an organism will be made in the light of the preferred processing conditions as well as the biochemical or biosynthetic capabilities of an organism having otherwise acceptable characteristics.

Preferred microorganisms for use in the above-mentioned process of the present invention include E. coli K-12 (JA 221) and especially E. coli K-12 (DS410). These microorganisms, JA 221 and DS410, are well known having the genotype F<sup>-</sup> thiA leuB6 trpE 5 recA and F<sup>-</sup> ara azi ton A lac Y min A min B rps L mal A xyl mtl thi respectively and are freely available to the public, having been freely available to the public since before 1 February 1985. Moreover E. coli JA 221 and E. coli DS 410 have been deposited by us, under the Budapest Treaty, with The National Collections of Industrial & Marine Bacteria Ltd, Torry Research Station, PO Box No. 31, 135 Abbey Road, Aberdeen, AB9 8DG Scotland under the deposition numbers NC1B 12099 and NC1B 12100 respectively. The deposition date in respect of each microorganism being

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7 June 1985.

Typically, such micro-organisms may be aerobically cultured in an aqueous medium containing as a source of assimilable carbon, together with necessary inorganic nutrients. Generally, where, for example, the interferon analogue is not passed out of the cell at a commercially useful rate, the organism may be cultured and harvested as the intact cell conveniently with subsequent extraction of the cells for example after separation from the medium. Where the interferon analogue is passed out of the cell into the surrounding culture solution, however, the analogue may be extracted therefrom in the conventional way. Such techniques are well known and require no further explanation.

According to a further feature of the present invention there is thus provided a transformant microorganism capable of expressing a polypeptide of formula I as hereinbefore defined, the said microorganism comprising a replicable plasmidic expression vehicle, which vehicle comprises genetic material coding for the said polypeptide.

The transformant microorganism is preferably an E. coli JA221 transformant, especially an E. coli DS410 transformant.

According to a further feature of the present invention there is provided a process for the preparation of a transformant microorganism as hereinbefore defined which comprises transforming a microorganism by the insertion therein of a replicable plasmidic expression vehicle, which vehicle comprises

genetic material coding for a polypeptide of formula I as hereinbefore defined.

Methods for the introduction of foreign genetic material into microorganisms have been widely described in the literature. Such methods comprise formation of a replicable plasmidic expression vehicle which vehicle comprises a vector and the foreign genetic material, and introduction of the vehicle into the microorganism. Introduction of the vehicle into the microorganism may be advantageously facilitated by subjecting the microorganism to an appropriate treatment, for example treatment with a calcium chloride solution. Where the genetically modified microorganism of the present invention comprises a methylotroph or an autotroph the replicable plasmidic expression vehicle comprising the gene of an interferon analogue of the present invention is preferably introduced thereto by transfer from another organism by cell conjugation, more preferably by transfer of the replicable plasmidic expression vehicle from a strain of E. coli.

According to a further feature of the present invention we provide a replicable plasmidic expression vehicle capable, in a transformant microorganism, of expressing a polypeptide of formula I as hereinbefore defined. We also provide according to a still further feature of the present invention a process for the preparation of such a replicable plasmidic expression vehicle which comprises inserting a gene coding for a polypeptide of formula I as hereinbefore defined or inserting a double stranded DNA fragment encoding a portion of the said polypeptide into a vector therefor at an appropriate insertion site whereby a replicable plasmidic expression vehicle is obtained capable of directing the synthesis of a polypeptide of formula I as hereinbefore defined in a transformant microorganism.

Thus in one embodiment of the present invention the replicable plasmidic expression vehicle may be prepared by inserting a gene coding for a polypeptide of formula I into a vector therefor. In a further embodiment a vector comprising a promoter sequence and a nucleotide sequence or nucleotide sequences which code for a portion of the polypeptide of formula I is used and the nucleotide sequence fragment(s) coding for the remainder of the polypeptide of formula I is (are) inserted into the vector to form the replicable plasmidic expression vehicle.

The replicable plasmidic expression vehicle of the present invention is advantageously prepared by the use of a plasmid vector comprising an E. coli lac or trp promoter, preferably a plasmid vector as described in European Patent Publication No. 0062971 the disclosure of which, as stated above, is for example incorporated herein by way of reference. Expression of the gene encoding for the polypeptide of formula I is for example effected under the influence of a trp promoter (the promoter of the E. coli tryptophan operon).

A synthetic trp promoter based on the tryptophan operon of E. coli K12 is conveniently employed and is present in the plasmid vector pSTP1 (Windass et al., Nucleic Acids Research, Vol. 10 (1983) p 6639) illustrated in Fig 2. Such a plasmid vector is of particular interest since it allows the gene coding for the polypeptides of formula I to be introduced between the ClaI and SalI sites of the plasmid vector just downstream of a potentially strong promoter.

The trp promoter together with such a gene (the Eco RI - SalI segment) may be removed from the vector and used as a portable expression unit, which unit constitutes a further feature of the present invention.

According to a further feature of the present invention there is provided a DNA sequence that encodes for a polypeptide of formula I as hereinbefore defined. Oligonucleotides selected from the aforesaid DNA sequence also constitute a further feature of the present invention.

The aforesaid DNA sequence and oligonucleotides may be prepared by any convenient process known per se, but one method which we have found advantageous for preparing the aforesaid DNA sequence and which constitutes a still further feature of the present invention comprises ligating appropriate oligonucleotides whereby to form a DNA sequence of the present invention.

The degeneracy of the genetic code permits substantial freedom in the choice of codons which can be used to construct a gene for the appropriate polypeptide of the present invention. Codons were normally chosen as those preferred in the expression of microbial genomes in a particular host such as E. coli. This consideration led to the preferred nucleotide sequences of the present invention which were selected to enable the polypeptides of the present invention to be expressed, such as the nucleotide sequence detailed in any one of the Examples hereinafter.

We have found that the synthesis of the gene of the polypeptides of the present invention may be facilitated by synthesizing oligonucleotides, for example comprising about 14 or 15 nucleotides, and then ligating oligonucleotides, for example 4-6 oligonucleotides, in the appropriate sequence to form fragments, which fragments may then be assembled to form sequences and the sequences ligated to form the gene.

The DNA sequence that encodes for the polypeptides of formula I is for example flanked by

appropriate initiation and termination codons and appropriate single stranded termini to allow ligation at a suitable site in a plasmid vector. The DNA sequence is thus preceded by for example:-

5

5' CGACGATG  
3' TGCTAC

10 which sequence comprises the initiation codon for methionine and the linker nucleotides which enable the DNA sequence to be inserted at a ClaI/TaqI restriction site. The DNA sequence may for example be followed by:-

15

5' TAAG  
3' ATTCAGCT

20 which sequence comprises a termination codon and the linker nucleotides which enable linkage to be effected to the vector for example at the SalI site. In the preparation of the DNA sequences of the present invention these sequences are preferably included in the appropriate 5' and 3' oligonucleotides where it is desired to prepare the DNA sequence by the above-described technique of ligating oligonucleotides to form sequences which are then ligated to form fragments and the fragments ligated to form the DNA sequence or gene.

25

30 We do not however exclude the possibility that alternative synthetic strategies may be used. For example, the degeneracy of the genetic code permits restriction sites to be present in the nucleotide sequence of the gene and this may be used to facilitate gene synthesis.

35 According to a further feature of the present invention we therefore provide a vector comprising a

promoter sequence and a nucleotide sequence or nucleotide sequences which code for a portion of the polypeptide of formula I, the vector being such that the nucleotide fragment coding for the remainder of the polypeptide of formula I may be inserted into the vector whereby to form replicable plasmidic expression vehicle of the present invention.

The nucleotide sequence of the vector at the site for insertion of the aforesaid gene fragment will thus be a restriction site(s) so that a restriction endonuclease may be used to cut the vector at the appropriate site for insertion therein of the said gene fragment. Thus, for example, the nucleotide sequence set out in Figure 1 has a Bst E II/Eca I restriction site (GGTAACC) at the codons for amino acids 44 to 46. Thus, where it is desired to effect changes to the IFN- $\alpha_2$  polypeptide within the first 43 amino acids, only the section of the gene spanning the restriction site at the 5' end of the gene and the Bst EII/Eca I restriction site need be prepared. This section of the gene or fragment is referred to herein as a Cla-BstE fragment. The replicable plasmidic expression vehicle may thus be prepared directly by inserting the appropriate Cla-Bst E fragment into a vector containing an appropriate promoter sequence, e.g. a trp promoter and the IFN  $\alpha_2$  gene sequence between the Bst EII/Eca I site and the 3' end of the gene at the appropriate site. The Cla-Bst E fragments may, for example, be prepared by the production of a DNA sequence, which contains the desired restriction sites, the sequence then being cleaved by digestion with the appropriate restriction endonuclease but, may also be prepared using modified terminal fragments so that the single-stranded cohesive end for Bst EII/Eca I is already present.

The use of restriction site(s) in this way thus enables the genes for a series of polypeptides, modified in a particular region, to be produced in a replicable plasmidic expression vehicle without the need for the total synthesis of each gene. Moreover several vectors spanning any combination of restrictions site(s) may be generated and modified genes prepared by such procedures can be used to generate any combination of these modifications by transferring restriction fragments between plasmidic vectors. By way of example restriction sites introduced into the IFN- $\alpha_2$  gene to facilitate modified gene synthesis are illustrated in the Table below:-

Oligonucleotide Changed	New sequence (5'-3')	Restriction site Introduced	New small sub-fragment
4 5	4 SauI CTAGGATGATTCG 5 SauI TCCTTAGTAAAGCT	SauI	A-SauI
29 30 31	29 XhoI AGCAGTAAAGCTCG 30 XhoI GCGGCACTGAGTCT 31 XhoI AGTGTGCAATGAGAC	XhoI	F-XhoI G-XhoI G-XhoI/SacI
35 36 37	35 SacI AAATTCATACCGAG 36 SacI TGGTAAAGCTGGTG 37 SacI CTCTACCGAGCACTG	SacI	G-SacI H-SacI H-SacI/XhoI
38 39 40	38 Xba I AGATGTTTCACTTC 39 Xba I AAAGATCTAAGAGC 40 Xba I ATGAGCGAGCTTCT	XbaI	H-XbaI
60 61 62	60 Bgl II ATCTCAGATTCAG 61 Bgl II ATGAGATCTTCAGC 62 Bgl II TGGACAGCGTAAAG	Bgl II	L-Bgl II M-Bgl II

The polypeptides of formula I as hereinbefore defined may be used as obtained or purified in a known or appropriate manner and formulated into pharmaceutical compositions for example by admixture with an appropriate inert or active diluent, which typically will be liquid, but may be solid.

The polypeptides of the present invention are potentially useful as antiviral, or anticancer agents or as modulators in the immune response of, for example, mammals and particularly man.

Various aspects of the present invention will now be described with reference to the following Examples which are illustrative of the invention.

#### Examples 1-31

##### Preparation of HuIFN-alpha<sub>2</sub> analogues.

##### A. Preparation of the genes of HuIFN-alpha<sub>2</sub> analogues.

IFN genes were prepared from oligodeoxyribonucleotides (oligonucleotides) according to the basic strategy outlined by Edge et al (Nature 1981, 292 756-762 and Nucleic Acid Res. 1983 11, 6419-6435) and in European Patent Publication No. 62971A2. These Examples describe the preparation of the genes of HuIFN-alpha<sub>2</sub> analogues which can be ligated to the plasmid vector pSTP-1, containing a synthetic trp promoter, to give expression of the gene in E. coli. 1.

##### Oligonucleotide synthesis

Oligonucleotides were prepared either by a solid-phase synthesis procedure as described in European Patent Publication No. 62971 A2 or by using the reagents and solvents described in the aforementioned patent application with the composite polydimethylacrylamide-Kieselguhr support in a continuous flow assembly apparatus as described by M. J. Gait et al. (J. Chem. Soc., Chem. Commun. 1982. p. 37-40). Crude

oligonucleotides were purified by ion-exchange and reverse-phase high-performance liquid chromatography essentially as described by C. R. Newton et al. (Anal. Biochem. 1983 129, 22-30).

5           For the purposes of these Examples the structure of the oligonucleotides referred to herein as 1 Taq, 2 Taq and by a number of from 3 to 68 are as identified by the horizontal arrows appearing above and below the relevant oligonucleotide sequence in Fig 1  
10       which depicts a synthetic interferon alpha<sub>2</sub> sequence. Where it is desired to refer to an oligonucleotide comprising an amino acid modification the same number will apply to the modified oligonucleotide as applied to the corresponding unmodified oligonucleotide with the  
15       proviso that additional notation is used to distinguish the modified oligonucleotide and its structure is identified hereinafter. It will further be noted that oligonucleotides 1 Taq and 2 Taq referred to herein differ from oligonucleotides 1 and 2 identified in Fig  
20       2 of European Patent Publication No. 62971A2 and the designation 1 Taq and 2 Taq has been used herein to avoid confusion. Thus oligonucleotides 1 Taq and 2 Taq possess the following sequences:-

25       1 Taq 5' CGACGATGTGTGAT 3'  
      2 Taq   3' TGCTACACACTAGACGGC 5'

      which sequences may be further modified if it is desired to code for amino acid(s) which differ from any of the  
30       first five amino acids of IFN-alpha<sub>2</sub> or if it is desired to omit any such amino acids.

2. Ligation of chemically synthesised oligonucleotides

      Oligonucleotides were assembled into small DNA sub-fragments A-M essentially as detailed in Table 1  
35       below:-

Table 1

	Fragments	Oligonucleotides
5	A	1-5
	B	6-10
	C	11-15
	D	16-20
10	E	21-25
	F	26-30
	G	31-35
	H	36-40
	I	41-45
15	J	46-50
	K	51-55
	L	56-60
	M	61-68

20

The oligonucleotides were assembled into the small DNA sub-fragments A-M either as described by Edge et al. (Nucleic Acids Res. 1983 11, 6419-6435) and in European Patent Publication No. 62971A2 or by the following modification as illustrated in the preparation of small sub-fragment B:-

Oligonucleotides 6,7,8 and 9 (400 pmol. each) (Fig. 1) were separately phosphorylated with polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP as described in the aforementioned European Patent Publication then mixed in the ratio 1:0.9:0.8:0.9 and ethanol precipitated. The precipitate was dissolved in water (50  $\mu$ l) and the solution heated at 100°C. for 2 minutes to destroy residual kinase activity. Oligonucleotide 10 (400 p mol.) was added and the mixture extracted with butanol and then lyophilised. The residue was annealed

and ligated to give fragment B as described in the  
aforementioned European Patent publication.

Small sub-fragment A was similarly prepared  
from oligonucleotides 1 Taq, 2 Taq, 3, 4, and 5 with  
5 oligonucleotide 1 Taq left unphosphorylated, to give a  
fragment subsequently referred to as A-taq.

### 3. Assembly of A to M into the Interferon Gene.

Sequences (I to III M) were constructed by  
similarly ligating fragments A-taq to M after  
10 phosphorylating the 5'-hydroxy termini of fragments B to  
L, with T4 polynucleotide kinase and ATP (containing  
0.15% of [ $\gamma$ - $^{32}$ P]ATP in groups as shown in Table 2.

Table 2

15

Sequence	Fragments
I	A-Ta <sub>q</sub> , B, C, D
II	E, F, G, H
III	I, J, K, L,
IIIM	I, J, K, L, M

20

The sequences I, II and III or IIIM were  
25 purified by gel electrophoresis in a 10% polyacrylamide  
slab and either I + II + III + M or I + II + IIIM  
ligated to give the gene of the appropriate  
Hu IFN- $\alpha_2$  analogue. After precipitation the product  
mixture was phosphorylated with T4 polynucleotide kinase  
30 as described previously.

The following HuIFN- $\alpha_2$  analogues were  
prepared by ligation of the oligonucleotides specified  
in the Table, according to the procedure described in  
this Example above and according to the basic strategy  
35 described in Edge et al (Nature 1981, 292, 756-762 and,  
Nucleic Acids Res. 1983 11, 6419-6435) and in European

Patent Publication No. 62971A2. As stated above the structure of the oligonucleotides referred to below as 1  
Taq, 2 Taq and by a number of from 3 to 68 are  
identified by the horizontal arrows above and below the  
5 oligonucleotide sequence in Fig 1. References herein to  
gamma interferon refer to the amino acid sequence  
detailed in "Structure of the human immune IFN gene"  
P W Gray or D V Goeddel Nature 298 859 (1982).

TABLE 3

		New oligonucleotide		New	
Ex	Name			Small sub	oligonucleotides
No.		Code	sequence (5'-3')	Fragment	used.
1.	endo-Asp <sup>43a</sup> -(Glu <sup>5</sup> , Asp <sup>10</sup> , Asn <sup>11</sup> ) IFN- $\omega_2$	1 taq	CGACGATGTCGAT	A-B1	1-taq, 2B1, x4, x5, x6
		2-B1	TCGGGCGAGTCACACATCTG	B-B1	x7, x8, 8, 9, 10
		x4	CTGCGGCGACACCAT		
		x5	CCAGGCTATGGGTC		
		x6	AGCCTGGATTAACGGT		
		x7	AGGGTAAAGACGTTAT		
		x8	CGTAACCTGATGCTG		

Ex	Name	New oligonucleotide		Rev	Small sub		oligonucleotides
		Code	sequence (5'-3')		Fragment	used	
No.							
1.		18F8	TTACGTCGACTCTTC	D-F8		16,17,18F8,19F8,20	
		19F8	TTGACGGTAACAGTTC				
2.	endo-Asp <sup>43e</sup> -[Leu <sup>151</sup> ,Arg <sup>160,163</sup> ]IFN- $\alpha_2$	F18 & F19	see Example 1	D-F8		16,17,18F8,19F8,20	
		x62	ATCGTTCCCTGCTT	M-B2		x2,x62,x63,x64,x65,x66,x67	
		x63	GGTTCATTAACACAGG				
		x64	TATCAACCAATCTTC				
		x65	AAAGTCTTGAGATT				
		x66	AAGAACGTTAAGGCGC				
		x67	TGACTTATTCCTTGCCCTT				
		x2	AAGCAATPAG				
3.	[Y(2-7) <sup>2-7</sup> ]IFN- $\alpha_2$	1C5	CGACATGCTTAC				
		12C5	CTGACGTACACATGCT	A-C5		1C5,2C5,3C5,4C5,5.	
		13C5	TGTCAGAACCATAT				
		14C5	CCAGCTTATATGGTTC				
4.	[Y(98-114)98-114 <sup>4</sup> ]IFN- $\alpha_2$	140C4	TTGCTCAGGCGCTTCC	H-C4		36,37,38,39,40C4.	
		141C4	CTGACCAACTACTCT	I-C4		41C4,42C4,43C4,44C4,45C4	
		142C4	TCACTAACAGGTAG	J-C4		46C4,47C4,48,49,50.	
		143C4	GTACTGACCTGAC				

Ex No.	Name	New oligonucleotide		New Small sub Fragments	oligonucleotides used.
		Code	sequence (5'-3')		
4.		44C4	CCCTGAGCTTCAGG		
		45C4	GTCCAGCCCAAGCT		
		46C4	GAATGATACCTTGG		
		47C4	ATCCATTCATCCCG		
5.	[Met <sup>100</sup> , Y(101-114) 101-114] IIRN- $\kappa_2$	41C6	TGCGTCATGTACTCT	I-C6	41C6, 42C6, 43C4, 44C4, 45C4.
		42C6	TCACTAACAGAGTAC	J-C4	Example 4 above.
6.	[Y(98-107) 98-107] IIRN- $\kappa_2$	44C7	AGCGAGTGTCCAGG	H-C4	See Example 4 above.
				I-C7	41C4, 42C4, 43C4, 44C7, 45.
7.	[Y(95-101) 101-107] IIRN- $\kappa_2$	41C8	TGCGTCATGTCAGG		
		42C8	GTCCATTTCTCCAGG		
		43C8	AAACTGACCAACTAC	I-C8	41C8, 42C8, 43C8, 44C8, 45.
		44C8	AGCGAGTGTAGTGG		

Ex	Name	New oligonucleotide		New	Small sub fragments	oligonucleotides
		Code	sequence (5'-3')			
8.	[K(2-7), Met <sup>100</sup> , Glu <sup>102</sup> , 103, Arg <sup>104</sup> , Gly <sup>106</sup> , Asn <sup>112</sup> , Ala <sup>113</sup> ] IFN- $\alpha_2$	1-4C5	see Example 3 for	A-C5		1C5, 2C5, 3C5, 4C5, 5.
			nucleotide sequence	I-C3		41C1, 42C1, 43C1, 44C1, 45C2.
		41 C1	TCGTCATGCAGAG			
		42 C1	ACA GTTCCTCTTC			
		43 C1	GAACTGTAGTGAG			
		45 C2	ACTCGCTGATGAAG			
		46 C2	GAGTCGTTCATC	J-C2		46C2, 47C2, 48, 49, 50
		47 C2	CAGACTCCATCCTG			
9.	[Y(2-7) Y(98-114)] IFN- $\alpha_2$	40-47C4	see Examples 4 and 3	A-C5		see Example 3 or 8.
		4 & 1-4C5	respectively for	H-C4		
			nucleotide sequences	I-C4		see Example 4
				J-C4		
10.	[Met <sup>100</sup> , Y(2-7), Y(100-114)] IFN- $\alpha_2$	1C5				
		2C5				
		3C5				
		4C5	see Examples 3,	A-C5		see Examples 3 or 8.
		41C6	4 & 5 for	I-C6		see Example 5.
		42C6	nucleotide sequences	J-C4		see Example 4.
		43-47C4				
11.	[Leu <sup>16</sup> , 21, 59, 111, 148] IFN- $\alpha_2$	712	CGCAACCTGCTCTG	B-12/3		6, 712, 812, 913, 1013
		812	TGGCGACACAGAGC			

Ex No.	Name	New oligonucleotide		New Small sub Fragment	oligonucleotides used.
		Code	sequence (5'-3')		
11.		913	CTCGCCCACTGC		
		1013	GAGTACGCGCAGT		
		2414	ATCAGTTCGTCAGT	B-14	21, 22, 23, 2414, 2514.
		2514	GACGATTCACAG		
		4515	ACTCCGCTGTAAG	I-15	41, 42, 43, 44, 4515.
		4615	GAGCTCTTTCAGC	J-15	4615, 47, 48, 49, 50.
		6016	AAOCACAGATTTCAG	L-16	56, 57, 58, 59, 6016.
		6116	CTCGTTCCTTCAGC	M-16	6116, 62, 63, 64, 65, 66, 67, 68.
12.	[Leu <sup>16</sup> ] IFN- $\alpha_2$	712	see Example 11 for nucleotide sequences.	B-12	6, 712, 812, 9, 10
13.	[Leu <sup>21</sup> ] IFN- $\alpha_2$	913	see Example 11 for nucleotide sequences	B-13	6, 7, 8, 913, 1013.
		1013			
14.	[Leu <sup>59</sup> ] IFN- $\alpha_2$	2414	see Example 11 for nucleotide sequences	E-14	see Example 11.
		2514			

Ex No.	Name	New oligonucleotide		New oligonucleotides	
		Code	sequence (5'-3')	Small sub Fragment	used
15.	[Leu <sup>111</sup> ] IFN- $\alpha_2$	4515	see Example 11 for nucleotide sequences	I-15 J-15	see Example 11.
16.	[Ala <sup>10,37,44,102,104</sup> ] IFN- $\alpha_2$	5D2 6D2 15D3 16D3 18D4 19D4 41D5 42D5-D6 43D6	AGCCTGCCCTAAGCCT AGGTTGCCAAGGCTAG CGCCATGACTTTGCT TGCCGGAAGCAAG TTAGCGAAGCTTTC TTGCTTAACCACTTC TGCGTATCCAGCT ACAGCAAGCCTGG GTTCCTGTAAACGAA	A-D2 B-D2 C-D3 D-D3/4 I-D5/6	1 Tag, 2 Tag, 3, 4, 5D2 6D2, 7, 8, 9, 10. 11, 12, 13, 14, 15D3. 16D3, 17, 18D4, 19D4, 20. 41D5, 42D5-D6, 43D6, 44, 45.
17.	[Ala <sup>137</sup> ] IFN- $\alpha_2$	55K10 56K10	AMGAATACAGCCT CAAGCGAAGCCTG	K-K10 L-K10	51, 52, 53, 54, 55K10. 56K10, 57, 58, 59, 60.
18.	[Ser <sup>29,138</sup> ] IFN- $\alpha_2$	12K1	AGAGAGGAGAACG	C-K1	11, 12K1, 13K1, 14, 15.

Ex No.	Name	New oligonucleotide		New small sub fragment	oligonucleotides used.
		Code	sequence (5'-3')		
18.		13K1	TCCTCTGAGAGAC	L-K1	56K1, 57K1, 58, 59, 60.
		56K1	CAGCAGAGGGCTG		
		57K1	TCCTCTGGAGATT		
19.	[Glu <sup>32</sup> ] IFN- $\gamma_2$	13K16	TCCTGCTGAGAGAG	C-K16	11, 12, 13K16, 14K16, 15.
		14K16	TCATGGGCGCTCTTTC		

Ex No.	Name	New oligonucleotide		New Small sub Fragment	oligonucleotides used
		Code	sequence (5'-3')		
20.	[Ala <sup>61,64,67</sup> ] IFN- $\alpha_2$	26G3	TTAGCAATCTGCGCA		
		27G3	ATTGCTTACCTGCT	F-G8	26G3, 27G3, 28G3, 29, 31
		28G3	TTAGTCTTACCCAGG		
21.	IFN- $\alpha_2$ (12-165)	5A8	CGACGATTCGGT	B-A8	5A8, 6A8, 7, 8, 9, 10 <sup>1</sup>
		6A8	AGGTCGACGACATCGT		
22.	IFN- $\alpha_2$ (10-165)	4A7	ACCCATCGT		
		5A7	CGACGATTCGGTACCCGT	B-A7	4A7, 5A7, 6A7, 8, 9, 10 <sup>1</sup>
		6A7	AGGTCGACGACGCT		

Ex No	Name	New oligonucleotide		New Small sub Fragment	oligonucleotides used
		Code	sequence (5'-3')		
23.	Met <sup>100</sup> Ges(101-110) IFN- $\alpha_2$	I	TGCTCAGTGTGAAG		oligonucleotide I used in place of small sub frag- ment I N.B also converts Ile 100 to Met 100
24.	[Ala <sup>10-15</sup> ] IFN- $\alpha_2$	5A11 6A11 7A11	AGCCTGCTGCAGCT GCAGCAGCAGCTGCAG GCTGCTGCAGTGCAG	A-A11 B-A11	1-Taq, 2-Taq, 3, 4, 5A11, 6A11, 7A11, 8, 9, 10
25.	[Phe <sup>115</sup> ] IFN- $\alpha_2$	46J2 47J2	AACTCTCTTCATC AAACTTCATCTG	J-J2	46J2, 47J2, 48, 49, 50

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Ex	Name	New oligonucleotide	New Small sub Fragment	oligonucleotides used.
No.		Code	sequence (5'-3')	
26.	IFN-2(4-155)	2A1	GGCATTGT	A-A1
		3A1	GGAGATGCGCAACTCAT	2A1, 3A1, 4, 5
		63A5	CTGTCCACTTAC	
		64A5	TGACTTACG	M-A5
				61, 62, 63A5, 64A5

Ex No.	Name	New oligonucleotide		New Small sub Fragment	oligonucleotides used.
		Code	sequence (5'-3')		
27.	[Ala <sup>32</sup> ] IFN- 2	13K12	TCCTGTCGAAAGCT	C-K12	11, 12, 13K12, 14K12, 15
		14K12	TCATGGCGAGCTTTC		
28	[Ile <sup>30</sup> ] IFN- 2	12K14	ATPACGGAGAACAGG		
		13K14	TCCTGTATCAAGAC	C-K14	11, 12K14, 13K14, 14K14, 15
		14K14	TCATGGCGGCTTTG		
29.	[Asn <sup>32</sup> ] IFN- 2	13K17	TCCTGTCTGAAAMC	C-K17	11, 12, 13K17, 14K17, 15
		14K17	TCATGGCGGCTTTC		
30	[Ala <sup>28</sup> ] IFN- 2	12K9	AGACACGAGAACAGG	C-K9	11, 12K9, 13K9, 14, 15
		13K9	GCTGTCTGAAAGAC		
31	[Glu <sup>5</sup> , Ser <sup>27</sup> , Met <sup>31</sup> , Leu <sup>59</sup> ] IFN	3C14	CTCAGCGAAACTAT )		1 Tag, 2 Tag, 3-C14,
		4C14SauI	CTAAGCAATGAGTTTC )	A-C14 Sau I	4-C15 Sau I, 5 Sau I
		5 Sau I	TCCTTACGTCACCGT )		
		11C15	GGTATCTCCCTGTCCT		
		12C15	AGACAGGAGAACAGG		
		13C16	TCCTGTCTGATGAC	C-C15/16	11-C15, 12-C15, 14-C16,
		14C16	TCATGGCGGCTTCATC		15
		2414	See Example 11 )	E-14	See Example 14
		2514	See Example 11 )		

1. In respect of this Example there is no small sub fragment A for the gene.

B. Cloning of the synthetic genes of  
HuIFN-alpha<sub>2</sub> analogues.

The synthetic genes of the interferon alpha<sub>2</sub> analogues listed in Table 3 were cloned into the plasmid vector, pSTP1 (Windass et al., Nucleic Acids Research, vol. 10, (1983) p6639), which included a promoter based on that of the tryptophan (trp) operon of E. coli K12.

For vector preparation, 200 µg. pSTP1 was dissolved in 168 µl. water, 2 µl. of 10 mg./ml. BSA and 20 µl. of 10 x restriction buffer (the restriction buffer is 6mM Tris. HCl pH 7.4, 6mM beta-mercaptoethanol 6mM MgCl<sub>2</sub>, 10 ml. of the restriction endonuclease, Cla I (New England Biolabs, 5 units/µl.) was added and the mixture was incubated at 37°C. for 3 hours or more until linearised plasmid was predominant over supercoiled and nicked circular forms.

Linearised plasmid was separated from undigested plasmid molecules on a 1.4% preparative agarose gel. The band of linear plasmid was electroeluted into 5 ml. of 1/10 concentrated tris-borate electrophoresis buffer (from a 1 to 100 dilution of 108 g Tris base, 55 g. boric acid and 20 ml. 0.5M EDTA (pH8) made up to 1 litre with water). The eluate volume was reduced to about 50 µl. by repeated butanol extractions and diluted to 500 µl. with water. The eluate was extracted twice with 500 µl. of phenol: chloroform (1:1), twice with 1 ml. of water saturated ether and then concentrated to 20 µl. by butanol extractions. To this was added sequentially 160 µl. water, 20 µl. 3M sodium acetate (pH5.2) and 500 µl. of ethanol. The linear plasmid was allowed to precipitate overnight at -20°C. and pelleted by centrifugation in an Eppendorf microfuge for 15 minutes. The pellet was taken up in 140 µl. water and 20 µl. of 10 x restriction

buffer was added followed by 20  $\mu$ l. of 1 mg./ml. BSA and 15  $\mu$ l. of 2M sodium chloride. 5  $\mu$ l. of the endonuclease SalI (Boehringer, 10 units/ml.) was added and the mixture was incubated at 37°C. for at least three hours until the large ClaI-SalI fragment of pSTP1 was predominant. This fragment was prepared as above from a 1.4% preparative agarose gel. The ClaI-SalI vector fragment was then taken up in 1 ml. water and the DNA concentration was measured and adjusted to 47  $\mu$ g./ml. For ligation, 14.9  $\mu$ l. of vector DNA (0.7  $\mu$ g.) was added to the synthetic interferon gene fragment (in water) and the DNA mixture was dried in vacuo. The DNA was taken up in 26.5  $\mu$ l. of a mixture containing 525  $\mu$ l. 2 x ligase buffer (132 mM Tris HCl, (pH 7.6), 13.2 mM  $MgCl_2$ ; 10 mM DTT and 0.4 mM ATP), 79  $\mu$ l. ligase diluent (50 mM Tris HCl, pH 7.6, 10mM 2-mercaptoethanol; 500  $\mu$ g./ml. BSA) 260  $\mu$ l. 10 mM ATP, 33.5  $\mu$ l. water and 30  $\mu$ l. T4 DNA ligase (BRL, 2.5 Weiss units/ $\mu$ l.). Ligation was for 2 hours at 5°C. and then 12°C. overnight.

10  $\mu$ l. of the DNA mixes were used directly to transform the strain JA221 using a modification of the standard procedure described by Mandel and Higa, Journal of Mol. Biol., 53 (1975) p154. The modifications involved the use of 0.1M  $CaCl_2$  in 5mM Tris. HCl pH8 as the transformation buffer, a 45 minute incubation of the cells on ice following addition of DNA, a 30 minute incubation on ice after heat shock treatment and subsequent addition of L broth to 3.8 ml. Incubations prior to plating were overnight at room temperature without shaking. Aliquots of suspensions were plated onto L plates with 50  $\mu$ g./ml. ampicillin. Transformants were screened for the presence of cloned synthetic genes of interferon analogues as follows:-

1. Colonies were streaked onto L plates with 10  $\mu$ g./ml. tetracycline in order to identify transformants including the intact vector plasmid, pSTP1 which

invariably did not include a gene for an interferon analogue.

2. Tetracycline sensitive transformants were analysed by colony hybridisation analysis using standard methods described in Molecular Cloning; A Laboratory Manual by Maniatis et al (Cold Spring Harbor).

For screening for recombinants including, for example, the synthetic interferon gene of Example 11, two separate hybridisations were performed using different small subfragments, one with a base sequence included in the natural interferon  $\alpha_2$  gene (group F), and the other with a sequence related to the synthetic interferon gene, for example to the synthetic interferon gene of Example 11 (subfragment BI2/3). 2 p moles of these subfragments were dried down and dissolved in 50  $\mu$ l. of a mixture composed of 80  $\mu$ l. of nick translation buffer (50 mM potassium phosphate pH 7.4, 5 mM  $MgCl_2$ ), 32  $\mu$ l. 200  $\mu$ M dATP, 32  $\mu$ l. 200  $\mu$ M dGTP, 32  $\mu$ l. 200  $\mu$ M dTTP, 25  $\mu$ l.  $\alpha^{32}P$  dCTP (10mCi./ml., 3,000Ci./mmol., Amersham), 579  $\mu$ l. water and 20  $\mu$ l. DNA polymerase I (12 units/ $\mu$ l. New England Biolabs). After 2 hours at room temperature, the labelled subfragments were isolated by Sephadex G<sup>50</sup>F chromatography using 10 mM Tris HCl. pH 8, 1 mM EDTA as running buffer. Lysis of colonies on filters and baking was performed as described in Molecular Cloning: A Laboratory Manual by Maniatis et al. (Cold Spring Harbor). Filters were prehybridised in 20  $\mu$ l. Denhardtts solution (a 50 fold dilution of Ficoll, polyvinyl pyrrolidone and BSA, each at 10 g./l. in water) at 65°C. for 1 hour. The  $^{32}P$  labelled subfragment probes were diluted into 2 volumes of 80% (v/v) formamide, 10 mM NaOH, 1mM EDTA, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) bromophenol blue and heated for 2 minutes at 100°C. before cooling on ice. The probe was added directly to the prehybridisation solution on

the filters, left to hybridise overnight at 65°C. and air dried before autoradiography. The recombinants, for example, of the interferon gene of Example 11 were identified by positive hybridisation to both group F and group BI 2/3 subfragments.

3. The correct organisation of the interferon analogue gene was checked by restriction analysis using endonuclease HpaII. Thus, for example, analysis on a 6% polyacryamide gel showed that the interferon gene gave a pattern of HpaII fragments similar to that of the interferon  $\alpha_2$  gene.

3 interferon recombinants giving positive hybridisation signals were grown up on a small scale in order to select a final clone expressing the interferon analogue of interest for subsequent use in production of the analogue. These small scale grows were conducted as follows:-

Recombinants were grown overnight (250 rpm in a New Brunswick G24 shaking incubator at 37°C.) in 10 ml. Spizizens medium (1/10 dilution of  $(\text{NH}_4)_2\text{SO}_4$  20 g./l.,  $\text{K}_2\text{HPO}_4$  106 g./l.,  $\text{Na}_3$  citrate 10 g./l.  $\text{KH}_2\text{PO}_4$  60 g./l.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  2g./l. pH 7.0 combined with 2/100 volume of a solution containing casein hydrolysate 25 g./l., L-leucine 1 g./l., thiamine 0.2 g./l., ampicillin 1.65 g./l. and glucose 100 g./l.) containing a high tryptophan concentration (100 mg./l.) and 50 ug./ml. ampicillin. Cells were pelleted and resuspended in 75 ml. Spizizens medium supplemented with a low tryptophan concentration of 2 mg./l. Incubation was for 5.5 hours at 37°C. at 250 rpm. Cells were pelleted at 4°C. and taken up in 2 ml. lysis buffer (15% sucrose, 50 mM. Tris. HCl. pH8 and 50 mM EDTA) and frozen overnight at -20°C. The cells were then thawed and 40  $\mu\text{l}$ . lysosyme (50 mg. per ml. of lysis buffer) was added followed by 20  $\mu\text{l}$ . 2.5% SDS. After incubation for 30 minutes on ice, 200  $\mu\text{l}$ . of DNAase solution (66  $\mu\text{g}$ ./ml. DNAase in

0.15 M Tris. HCl. pH 7.5, 0.28 M  $MgCl_2$ , 40 mM  $CaCl_2$ ) was, added and the lysate was incubated for 30 minutes on ice. The lysate was then sonicated for 90 seconds using the cup tip of a Heat Systems W-375 Sonicator set at full power. The lysate solution was clarified by centrifugation in a Sorvall SS34 rotor at 15,000 rpm for 20 minutes at 4°C. The supernatant was decanted and aliquoted for biochemical and biological evaluation of the interferon analogue.

C. 1. Example of Production of Interferon Analogue Proteins in E. coli JA221.

A recombinant strain of E. coli JA221 expressing an IFN  $\alpha_2$ -related protein was recovered from stock, streaked on an L Agar plate supplemented with ampicillin (50  $\mu g./ml.$ ) and incubated at 37°C. for 17 hours. A loop of cells was transferred from this plate into each of 4 x 250 ml. Erlenmeyer flasks containing 75 ml. Spizizen Medium 1, and these flasks shaken at 300 rpm on an orbital incubator at 37°C., overnight. Bacterial cells were harvested by centrifugation in 6 x 37.5 ml. aliquots in the SS34 rotor of a sorval RC5B centrifuge at 10,000 rpm for 6 minutes at room temperature, and resuspended in 3 ml. of prewarmed Spizizen Medium 2. These resuspended cells were then added to 6 x 750 ml. aliquots of Spizizen Medium 2 in 2L Erlenmeyer flasks and shaken at 250 rpm on an orbital incubator at 37°C. for 5.5 hours. At the end of this time, bacterial cells were harvested by centrifugation in the H6000A rotor of a Sorval RC3B centrifuge at 5,000 rpm for 20 minutes at 4°C, and resuspended in a total volume of 40 ml. lysis buffer (50mM EDTA, 15% (w/v) sucrose in 50 mM Tris-HCl pH 8.0). The resultant 40 ml cell suspension was stored at -20°C. prior to lysis.

Media

Spizizen Medium 1 contained per litre of distilled water:  $(NH_4)_2SO_4$ : 2.0 g;  $KH_2PO_4$ : 10.6 g;

Na<sub>3</sub> citrate: 1.0 g; KH<sub>2</sub>PO<sub>4</sub>: 6.0 g; MgSO<sub>4</sub> · 7H<sub>2</sub>O: 0.2 g;  
casein hydrolysate: 0.5 g; L-leucine: 20 mg; thiamine:  
4 mg; L-tryptophan: 100 mg; ampicillin: 33 mg;  
glucose: 2 g.

- 5                Spizizen Medium 2 was identical except that it  
contained L-tryptophan at 2 mg./l. and did not contain  
ampicillin.

              L Medium contained per litre of distilled  
water: Bacto tryptone: 10 g; Bacto Yeast Extract: 5 g;  
10    NaCl: 5 g; Glucose 1 g; and when necessary, Bacto Agar:  
15 g.

2.    Production of Interferon Analogue Proteins in  
      E. coli DS410

- A plasmid preparation from the recombinant  
15    strain of E. coli JA221 referred to in B above is used  
to transform E. coli DS410 and the transformant, which  
expresses an IFN alpha<sub>2</sub> protein analogue, was streaked  
on an L Agar plate supplemented with ampicillin (50  
µg./ml.) and incubated at 37°C. for 17 h. A loop of  
20    cells were transferred from this plate into each of 2 x  
250 ml. Erlenmeyer flasks containing 75 ml. of L-broth  
supplemented with ampicillin (50 µg./ml.), and these  
flasks shaken at 300 rpm on an orbital incubator at  
37°C, overnight. Bacterial cells were harvested by  
25    centrifugation in 4 x 37.5 ml. aliquots in the SS34  
rotor of a Sorval RC5B centrifuge at 10,000 rpm for 6  
minutes at room temperature, and resuspended in 3 ml.  
prewarmed Spizizen Medium 2. These resuspended cells  
were pooled in 63 ml. prewarmed Spizizen Medium 2, and  
30    3.75 ml of the resultant suspension inoculated into each  
of 12 x 75 ml aliquots of Spizizen Medium 2 in 250ml.  
Erlenmeyer flasks which were subsequently shaken at 300  
rpm on an orbital incubator at 37°C. for 7h. At the end  
of this time bacterial cells were harvested and  
35    processed as described in C1.

D.    Lysis of Bacteria and Extraction of Interferon

### Analogue Proteins.

40 ml. of a frozen cell suspension was thawed to 4°C. and 800 µl. of a solution of lysozyme in lysis buffer (50 mg./ml.) added, immediately followed by 400 µl. LDS in distilled water (2.5% w/v). After incubation on ice for 30 minutes, 4 ml. DNase solution (ca. 5 µg. DNase dissolved in 150 mM Tris. HCl. pH 7.5 containing 280 mM MgCl<sub>2</sub> and 4 mM CaCl) was added and incubation on ice continued for a further 30 minutes. The resultant viscous material was subject to ultrasonic disintegration by 3 x 30s pulses from a 150 W MSE ultrasonic disintegrator set to deliver full power, and then centrifuged at 15,000 rpm for 15 minutes at 4°C. in the SS34 rotor of Sorval RC5B centrifuge. The supernatant from this centrifugation was then further centrifuged at 40,000 rpm for 1h. at 4°C. in the 50.2 Ti rotor of a Beckman L5-50B ultracentrifuge. The supernatant from this centrifugation contained the interferon analogue protein and was further purified as required.

### E. Purification

#### 1. Purification by precipitation and size exclusion chromatography

All operations were at 4°C. unless indicated otherwise.

Clarified supernatant (40 ml.) containing the interferon analogue was prepared as hereinbefore described.

Trichloroacetic acid (50% v/v, 2 ml.) was added dropwise with stirring to give a final concentration of 2.5%. (Alternatively saturated ammonium sulphate solution (80 ml.) was added dropwise with stirring over 15 minutes to give a final concentration of 50%). After stirring for 30 min and standing for 30 min, the precipitated protein was collected by centrifugation at 10,000 rpm for 30 min in

an MSE High Speed 18 centrifuge.

5       The supernatant was discarded and the pellet  
extracted into 0.1M  $K_2HPO_4$ , pH8 (20 ml.) using a Ystral  
homogeniser. The milky suspension was adjusted to pH8  
10 by dropwise addition of 2M NaOH with stirring using a  
Radiometer pH meter 26. The extract was  
ultracentrifuged at 34,000 rpm for 30 min in a 50.2.Ti  
rotor in a beckman L5-50B ultracentrifuge and the  
supernatant (21 ml.) applied immediately to a column  
15 (5 x 90 cm.) of Ultrogel Aca54 equilibrated in 0.1M  
 $K_2HPO_4$ , pH8 at 7°C. The column was eluted with the same  
buffer at a flow rate of 40 ml./hr. and fractions of  
Diaflo cell using an Amicon YM10 membrane. The  
20 retentate was filtered through a Millex GV, 0.22  $\mu$ m.  
filter unit and the sterile filtrate (19 ml.) was  
divided into multiple aliquots and stored at -20°C; a  
new aliquot was used for each assay approximately 12  
ml. were collected. Fractions 95-125, known to contain  
the interferon analogue and excluding over 80% of total  
bacterial protein, were pooled (400 ml.) and  
concentrated to about 20 ml. in an Amicon

25       Purification was monitored by antiviral and  
NK2-IRMA assays and the antiviral and NK2-IRMA titres  
for each relevant analogue are set out in Table 4 below.  
A hyphen in the NK2-IRMA assay column indicates that the  
relevant analogue was inactive in the NK2-IRMA assay.

TABLE 4

## E. Gel filtration purified analogue profiles

Example No.	Protein	Profile Sample Titre (U/ml.)	
		AV ( $\times 10^6$ )	NK-21RPA ( $\times 10^6$ )
3.	[ (2-7) <sup>2-7</sup> ] IFN-2	0.26	3.9
4.	[ (98-114) 98-114 ] IFN-2	0.42	—
5.	[ Met <sup>100</sup> , (101-114) 101-114 ] IFN-2	0.09	—
6.	[ , (98-107) 98-107 ] IFN-2	0.22	5.3
8.	[ (2-7) , Met <sup>100</sup> , Glu <sup>102</sup> , 103 , Arg <sup>104</sup> , Gly <sup>106</sup> , Asn <sup>112</sup> , Ala <sup>113</sup> ] IFN-2	0.09	—
9.	[ (2-7) , (98-114) ] IFN-2	0.21	—
10.	[ Met <sup>100</sup> , (2-7) , (101-114) ] IFN-2	0.05	—
11.	[ Leu <sup>16</sup> , 21 , 59 , 111 , 148 ] IFN-2	0.02	0.36
12.	[ Leu <sup>16</sup> ] IFN-2	0.54	3.9
13.	[ Leu <sup>21</sup> ] IFN-2	0.28	9.5
14.	[ Leu <sup>59</sup> ] IFN-2	0.02	0.35
15.	[ Leu <sup>111</sup> ] IFN-2	0.59	1.4
22.	[ IFN-2 (4-155) ]	0.39	0.5

The interferon analogue protein still represents only about 0.1% of the total protein in the bioprofiling sample and as such cannot be distinguished directly by staining after polyacrylamide gel electrophoresis. The analogue protein was characterised by transforming the mini-cell forming strain E. coli DS410 with the expression plasmid as described in D. Plasmid genes are expressed selectively in minicells allowing the proteins encoded by those genes to be radiolabelled selectively. Thus minicells were labelled with  $^{35}\text{S}$ -methionine and the interferon analogue was characterised by polyacrylamide gel electrophoresis in the presence of SDS run under reducing conditions and revealed by fluorography.

2. Purification by antibody affinity chromatography  
a) on NK-2 Sepharose.

Clarified supernatant (33 ml.) containing the interferon analogue was prepared as hereinbefore described and was applied at a flow rate of about 10 ml./hr. to a column (200  $\mu\text{l}$ . bed volume) of NK2-sepharose monoclonal antibody immuno-adsorbent support (Celltech Ltd, Slough, UK) equilibrated at room temperature in phosphate buffered saline (PBS) as recommended by the manufacturer. After all the sample was loaded the column was washed with PBS (2 x 1 ml; 1 x 40 ml) and the interferon analogue eluted with 0.1M citric acid - 0.3M NaCl (pH2). The fraction (400  $\mu\text{l}$ .) containing the analogue was adjusted to pH6 by addition of 450  $\mu\text{l}$ . of 0.25M mixed phosphate buffer, pH 7.5 (0.25M  $\text{NaH}_2\text{PO}_4$ -0.25M  $\text{Na}_2\text{HPO}_4$ , 1:5). This solution was diluted five-fold and twenty-fold with RPM1-1640 medium containing 10% foetal calf serum, 2mM glutamine, 2mM sodium pyruvate, 1% vitamins, 50  $\mu\text{g}$ .  $\text{ml}^{-1}$  gentamycin and  $5 \times 10^{-5}\text{M}$  2-mercaptoethanol to give samples ready for bioprofiling. These samples were divided into

multiple aliquots and stored at  $-20^{\circ}$ ; a new aliquot was used for each assay.

The purification was monitored by antiviral and NK2-IRMA assays and the final product was characterised by polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions.

b. on YOK-Sephadex

Clarified supernatant (33 ml.) containing the interferon analogue was prepared as hereinbefore described and was applied at a flow rate of about 10 ml./hr. to a column (200  $\mu$ l. bed volume) of YOK-Sephadex monoclonal antibody immuno-absorbent support (Celltech Ltd, Slough, UK) equilibrated at room temperature in phosphate buffered saline (PBS) as recommended by the manufacturer. After all the sample was loaded the column was washed with PBS (2 x 1 ml; 1 x 40 ml.) and the interferon analogue eluted with 0.1M citric acid - 0.3M NaCl (pH2). The fraction (400  $\mu$ l.) containing the analogue was adjusted to pH6 by addition of 450  $\mu$ l. of 0.25M mixed phosphate buffer, pH 7.5 (0.25M  $\text{NaH}_2\text{PO}_4$ -0.25M  $\text{Na}_2\text{HPO}_4$ , 1:5). This solution was diluted five-fold and twenty-fold with RPMI-1640 medium containing 10% foetal calf serum, 2mM glutamine, 2mM sodium pyruvate, 1% vitamins, 50  $\mu$ g.  $\text{ml}^{-1}$  gentamycin and  $5 \times 10^{-5}$ M 2-mercaptoethanol to give samples ready for bioproliferation. These samples were divided into multiple aliquots and stored at  $-20^{\circ}\text{C}$ .; a new aliquot was used for each assay.

The purification was monitored by antiviral and NK2-IRMA assays and the final product was characterised by polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions.

The antiviral and NK2-IRMA titres for each relevant analogue are set out in Table 5 below. A hyphen in the NK2-IRMA column indicates no cross-reactivity. The antiviral and NK2-IRMA titres for the analogues of Examples 27-30 were determined at the clone selection stage.

TABLE 5

Monoclonal antibody purified analogue profiles

Example No.	Protein	Profile Sample Titre (U/ml.)	
		AV ( $\times 10^6$ )	NC-2IRMA ( $\times 10^6$ )
1	endo-Asp <sup>43a</sup> -[Glu <sup>5</sup> , Asp <sup>10</sup> , Asn <sup>11</sup> ] IFN- $\alpha_2$	6.3	61
2	endo-Asp <sup>43a</sup> -[Leu <sup>151</sup> , Arg <sup>160</sup> , 163] IFN- $\alpha_2$	58.0	160
3	[Y(2-7)] <sup>2-7</sup> IFN- $\alpha_2$	1	150
4	[Y(98-114) 98-114] IFN- $\alpha_2$	6.1	-
5	[Met <sup>100</sup> , Y(101-114) 101-114] IFN- $\alpha_2$	0.7	-
6	[Y(98-107) 98-107] IFN- $\alpha_2$	11.2	130
7	[Y(95-101) 101-107] IFN- $\alpha_2$	8.0	36
8	[Y(2-7), Met <sup>100</sup> , Glu <sup>102</sup> , 103, Arg <sup>104</sup> , Gly <sup>106</sup> , Asn <sup>112</sup> , Ala <sup>113</sup> ] IFN- $\alpha_2$	3.1	-
9	[Y(2-7), Y(101-114)] IFN- $\alpha_2$	4.7	-
10	[Met <sup>100</sup> , Y(2-7), Y(101-114)] IFN- $\alpha_2$	1.0	-

Continuation....(2)

TABLE 5

Example No.	Protein	Profile Sample Titre (U/ml.)	
		AV ( $\times 10^6$ )	NK-2IRRA( $\times 10^6$ )
11.	[Leu <sup>16,21,59,111,148</sup> ] IFN- $\alpha_2$	1.0	13
12.	[Leu <sup>16</sup> ] IFN- $\alpha_2$	5.5	27
13.	[Leu <sup>21</sup> ] IFN- $\alpha_2$	5.1	64
14.	[Leu <sup>59</sup> ] IFN- $\alpha_2$	13.8	86
15.	[Leu <sup>111</sup> ] IFN- $\alpha_2$	6.2	18
16.	[Ala <sup>10,37,44,102,104</sup> ] IFN- $\alpha_2$	19.7	74
17.	[Ala <sup>137</sup> ] IFN- $\alpha_2$	30	-
18.	[Ser <sup>29,138</sup> ] IFN- $\alpha_2$	0.18	5.5
19.	[Glu <sup>32</sup> ] IFN- $\alpha_2$	5	41
20.	[Ala <sup>61,64,67</sup> ] IFN- $\alpha_2$	0.02	0.29
21.	IFN- $\alpha_2$ (12-165)	0.004	0.03

Continuation....(3)

TABLE 5

Example No.	Protein	Profile Sample Titre (U/ml.)	
		AV (x 10 <sup>6</sup> )	NR-217RA (x 10 <sup>6</sup> )
22.	IFN- $\alpha_2$ (10-165)	0.003	0.01
23.	Met <sup>100</sup> des (101-110) IFN- $\alpha_2$	1.0	3.8
24.	[Ala <sup>10-15</sup> ] IFN- $\alpha_2$	0.04	11
25.	[Phe <sup>115</sup> ] IFN- $\alpha_2$	0.07	0.15
26.	IFN- $\alpha_2$ (4-155)	1.6	6.4
27.	[Ala <sup>32</sup> ] IFN- $\alpha_2$	0.009	1.1
28.	[Ile <sup>30</sup> ] IFN- $\alpha_2$	0.01	2.9
29.	[Asn <sup>32</sup> ] IFN- $\alpha_2$	0.04	0.8
30.	[Ala <sup>28</sup> ] IFN- $\alpha_2$	0.14	2.2
31.	[Glu <sup>5</sup> , Ser <sup>27</sup> , Met <sup>31</sup> , Leu <sup>59</sup> ] IFN- $\alpha_2$	1.3	310

**Example 32**

This Example illustrates the preparation of [Leu<sup>16,21,59,111,148</sup>]IFN- $\alpha_2$ . The gene for this protein is hereinafter referred to for convenience as I-1. The procedure described in Examples 1 - 31 was repeated with oligonucleotides 7,8,9,10,24,25,45,46,60 and 61 in Figure 1 replaced by oligonucleotides 7-I2, 8-I2, 9-I3, 10-I3, 24-I4, 25-I4, 45-I5, 46-I5, 60-I6 and 61-I6 (Table 6).

**TABLE 6**

	Oligonucleotide	Sequence (5'-3')	Amino Acid	New
			changed	Amino Acid
15				
	7-12	CGCACCCTGCTCCTG	16	Leu
	8-12	TGGGCCAGCAGGAGC		
20	9-13	CTGGCCCAACTGCGC		
	10-I3	GAGATACGGCGCAGT	21	Leu
	24-I4	ATCAGTTCGTGCAGT	59	Leu
	25-I4	GAAC TGATTCAACAG		
	45-I5	ACTCCGCTGCTGAAAG	111	Leu
25	46-I5	GAGTCTTCTTCAGC		
	60-I6	AACGCAGGATTCAG	148	Leu
	61-I6	CTGCGTTCCTTCAGC		

Fragments A to M were prepared by ligating oligonucleotide mixtures according to the strategy outlined previously. In this Example, fragments B-I 2/3 E-I4, I-I5, J-I5, L-I6, and M-I6 replace fragments B,E,I,J,L and M in Examples 1-31.

Sequences I, II and IIIM were constructed from the fragments shown in Table 7 after phosphorylation of the 5'-hydroxy termini of fragments B to L.

TABLE 7

Sequence	Fragments used
I(I-1)	A-Taq, B-I 2/3, C,D
II (I-1)	E-I4, F,G,H
IIIM (I-1)	I-I5, J-I5, K, L-I6, M-I6

The gene I-1 was prepared by ligating sequences I(I-1), II(I-1) and IIIM(I-1) and phosphorylated with T4 polynucleotide kinase.

The gene I-1 was cloned as described in Section B of Examples 1-31, and expressed in E.coli JA 221 as described in Section C1, the interferon analogue was extracted by lysis of the bacteria as described in Section D and purified by precipitation and size exclusion chromatography as described in Section E of the aforesaid Examples.

The purification was monitored by antiviral and NK2-IRMA assays and the final product characterised by polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions. The titres and recoveries for [Leu<sup>16,21,59,111,148</sup>]IFN- $\alpha_2$  are summarized below:-

TABLE 8

Sample	Vol.	Antiviral activity		NC2-IRNA activity	
		units/ml.	total units	units/ml.	total units
lysate supernatant	40	$1.3 \times 10^4$	$5.2 \times 10^5$	$4.0 \times 10^5$	$1.6 \times 10^7$
extracted protein pellet	21	N/D	—	$3.7 \times 10^5$	$7.8 \times 10^6$
final sterile filtrate	19	$2.3 \times 10^4$	$4.4 \times 10^5$	$3.6 \times 10^5$	$6.8 \times 10^6$
recovery	—	858		438	

Example 33Preparation of [Ala<sup>10-15</sup>]IFN- $\alpha_2$ A. Oligonucleotide synthesis

The procedure described in Examples 1-31 was repeated using as the new oligonucleotide sequences 5-All, 6-All and 7-All instead of the oligonucleotides 5,6 and 7 respectively. The sequences of 5-All, 6-All and 7-All are as follows:-

	<u>oligonucleotide</u>	<u>Sequence (5'-3')</u>
10	5-All	AGCCTGGCTGCAGCT
	6-All	GCAGCAGCAGCTGCAG
	7-All	GCTGCTGCGATGCTG

15 Fragments A-taq to D were prepared by ligating appropriate oligonucleotide mixtures. In this example, fragment A-All replaces A-Taq and B-All replaces B. A-All comprises oligonucleotides 1 Taq, 2-Taq, 3,4 and 5-All and B-All comprises oligonucleotides 6-All, 7-All, 20 8, 9 and 10. Ligation of fragments A-All, B-All, C and D generates a DNA sequence equivalent to sequence I in Examples 1-31 and includes the Bst E II/Eca I restriction site. Following isolation of the A-D fragment from a polyacrylamide gel it was ethanol precipitated and resuspended in water (37.75  $\mu$ l.). 25 Bovine serum albumin (5  $\mu$ g. in 5  $\mu$ l), Bst E II (3 units in 1  $\mu$ l.), a buffer (0.6M Tris HCl pH 7.4, 0.6 M  $MgCl_2$  and 0.6M mercaptoethanol) (5  $\mu$ l) and 2M NaCl (1.25  $\mu$ l.) were added and the mixture incubated at 37°C. for 16 30 hours. The restriction digests were stopped by addition of 3M NaCl, pH 5.6 (20  $\mu$ l.) and water (130  $\mu$ l.) and the DNA precipitated with ethanol then fractionated in a 10% polyacrylamide non-denaturing gel. The most-slowly migrating band was eluted from the gel, ethanol 35 precipitated and 5' phosphorylated to give the Cla-Bst E fragment.

### B. Cloning of the [Ala<sup>10-15</sup>]IFN- $\alpha_2$ gene

The vector for cloning the [Ala<sup>10-15</sup>]IFN- $\alpha_2$  gene consisted of the large ClaI-SalI fragment of the plasmid pSTP1 (see examples 1-31) ligated to the small BstEII-SalI interferon gene fragment of the vector pIFS1205 (Edge et al., Nucleic Acids Research, Volume 11 (1983) p 6419). Fragments were isolated as for Examples 1-31 and ligated together prior to reisolation from an agarose gel as above. The DNA concentration was adjusted to 10  $\mu\text{g./ml.}$  and mixed with a sample of the synthetic [Ala<sup>10-15</sup>]IFN- $\alpha_2$  gene fragment. The mixture was dried and taken up in 30  $\mu\text{l.}$  of a mixture containing 65  $\mu\text{l.}$  10 x ligase buffer, 65  $\mu\text{l.}$  1 mg./ml. BSA, 510  $\mu\text{l.}$  water and 10  $\mu\text{l.}$  T4 DNA ligase (Boehringer, 1.8 units/ $\mu\text{l.}$ ). Incubations were for 1 hour at 4°C. and overnight at 12°C. Ligations were stopped by heating to 65°C. for 15 minutes. 6  $\mu\text{l.}$  of the ligation mixes were used directly to transform the strain MM294 by the method described by Hanahan (Jour. of Mol. Biol 166 (1983)p.557). Screening for [Ala<sup>10-15</sup>]IFN- $\alpha_2$  gene recombinants by colony hybridisation was as described for Examples 1-31 using nick translated probes for small subfragments as described in Examples 1-31.

In addition, a colony hybridisation analysis was performed with oligonucleotide probes. For screening for [Ala<sup>10-15</sup>]IFN- $\alpha_2$  gene recombinants, the 16 base long oligonucleotide 6-All was used.  $5 \times 10^{-4}$  OD units of oligonucleotide was dried in vacuo and taken up in 20  $\mu\text{l.}$  of a mixture containing 25  $\mu\text{l.}$  <sup>32</sup>P ATP (10mCi/ml, 5000 ci/mmol, New England Nuclear), 50  $\mu\text{l.}$  10 x kinase buffer (0.5M Tris pH9, 10mM EDTA), 50  $\mu\text{l.}$  1mM spermidine, 50  $\mu\text{l.}$  0.2M DTT, 50  $\mu\text{l.}$  100mM MgCl<sub>2</sub> 5  $\mu\text{l.}$  T4 polynucleotide kinase (2 units/ $\mu\text{l.}$ , New England

Biolabs) and 270  $\mu$ l. water. The labelled oligonucleotide was diluted into 10 ml. 6 x SSC for hybridisation. The hybridisation temperature used was guided by the formula  $4(G/C)+2(A/T)^{\circ}C$ . where G/C and A/T denote the number of guanine/cytosine or adenine/thymine nucleotides in the oligonucleotide. Thus, for the probe 6-All G/C=11, A/T=5), the formula yields the result  $54^{\circ}C$ ., the hybridisation actually being performed at  $56^{\circ}C$ . for at least 1 hour and filters were subsequently air dried for autoradiography. [Ala<sup>10-15</sup>]IFN- $\alpha_2$  recombinants were identified by positive hybridisation signals. The integrity of the [Ala<sup>10-15</sup>]IFN- $\alpha_2$  analogue gene was checked by Hpa II restriction analysis as for Examples 1 - 31. Lysis of recombinants for biochemical and biological evaluations of interferon were as described in Examples 1 - 31.

The vector containing the [Ala<sup>10-15</sup>]IFN- $\alpha_2$  gene was expressed in E.coli DS410 as described in Section D3 of Examples 1-31, the bacteria lysed as described in Section C and [Ala<sup>10-15</sup>]IFN- $\alpha_2$  purified by antibody affinity chromatography on NK2-Sepharose. The purification was monitored by antiviral and NK2-IRMA assays and the final product characterized by polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions. The titres and recoveries for [Ala<sup>10-15</sup>]IFN- $\alpha_2$  are summarized below:-

TABLE 9

Sample	Vol.	Antiviral Activity		NK2-IRMA Activity	
		units/ml.	total units	units/ml.	total units
lysate supernatant	36	$2.1 \times 10^3$	$7.6 \times 10^6$	$4.9 \times 10^5$	$1.8 \times 10^7$
column eluate pH6	0.85	$3.2 \times 10^4$	$2.7 \times 10^8$	$1.1 \times 10^7$	$9.4 \times 10^6$
recovery			368		528

Example 34

The procedure of Example 33 was repeated except that the oligonucleotides 17 and 18 were replaced by the oligonucleotides 17-Bst E and 18-BstE:-

5

Oligonucleotide	Sequence (5'-3')
17-Bst E	TTCCCGCAGGAAGAGTTCG
18-Bst E	GTTACCGAACTCTTCC

10

Fragment D was thus replaced by fragment D-Bst E, prepared from oligonucleotides 16, 17-Bst E and 18-Bst E. Ligation of A-taq, B, C-K11 and D-Bst E, followed by phosphorylation of the 5' ends with T4 polynucleotide kinase and ATP and isolation of the product by

15

gel-electrophoresis gives the Cla-BstE fragment.

The use of the modified fragment D ensures that the single-stranded cohesive end for Bst E II/Eca I is present.

20

Example 35

The procedure described in Examples 1 to 31 was repeated in order to prepare the analogue of Example 21 except that oligonucleotides 4 and 5 as detailed below were replaced by alternative sequences which introduced restriction enzyme sites, but retained the protein sequence of the interferon analogue.

25

Example 49TABLE 10

Oligonucleotide Changed	New sequence (5'-3')	Restriction Site Introduced	New small sub-fragment
4	4 Sau I CTAGGAATGAGTTG	Sau I	A-Sau I
5	5 Sau I TCCTTAGGTAGCCGT		

Analogues of the aforementioned Examples were tested for antiviral and antiproliferative activity in the following assays:-

(i) Antiviral assay

5                   Antiviral titers were determined in cytopathic effect inhibition assays by a dye uptake method, using the human amnion cell line WISH challenged with encephalomyocarditis virus. The assay was calibrated using the NIH human leukocyte IFN standard,  
10                   G-023-901-527. An internal standard was employed in every assay.

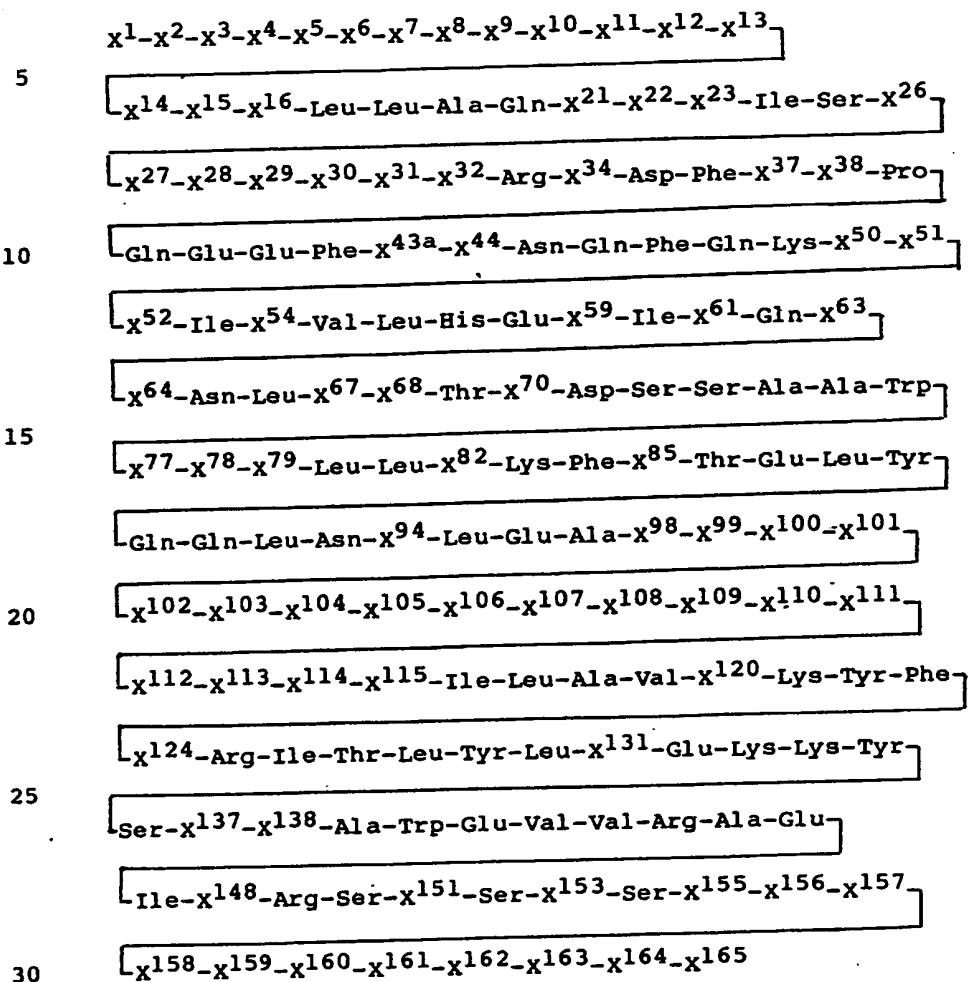
(ii) Growth inhibition assay

                  The Daudi cell line was grown in RPMI-1640 medium supplemented with 20mM HEPES and 10% FCS.  
15                   Cultures containing  $5 \times 10^4$  Daudi cells in 100  $\mu$ l. of complete medium were incubated with 100  $\mu$ l. of medium containing 0 to 1000U. of IFN for 72 hr. at 37°C. Four replicates were used for each sample dilution.  
                  [<sup>3</sup>H]Thymidine (1 $\mu$ Ci in 50  $\mu$ l.; 5 Ci/mmol) was added,  
20                   and the cultures were incubated for a further 24 hr. Cultures were subsequently processed using an automated cell harvester. Results were expressed as the percentage inhibition of [<sup>3</sup>H]-thymidine uptake of test sample compared with a medium control.

25                   The tested analogues were all found to possess antiviral activity and the analogues of Examples 2, 4, 5, 11, 16, 18 and 26 were in particular found to possess a ratio of antiviral to antiproliferative activity (AV/AP) substantially better than the AV/AP of 3  
30                   reported in the literature for natural human IFN- $\alpha_2$ .

Claims

1. A polypeptide of the formula I:-



in which  $x^1, x^2, x^3, x^4, x^5, x^6, x^7, x^8, x^9, x^{10}, x^{11}, x^{155}, x^{156},$

35  $x^{157}, x^{158}, x^{159}, x^{160}, x^{161}, x^{162}, x^{163}, x^{164}$  and  $x^{165}$  may  
 each represent a naturally occurring alpha-amino acid  
 and  $x^{12}$  represents Arg or Ala,  $x^{13}$  represents Arg or

Ala,  $x^{14}$  represents Thr or Ala,  $x^{15}$  represents Leu or Ala,  $x^{16}$  represents Met, Leu or Ile,  $x^{21}$  represents Met or Leu,  $x^{22}$  represents Ser, Arg or Gly and  $x^{23}$  represents Arg or Lys,  $x^{26}$  represents Leu or Pro,  $x^{27}$  represents Ser or Phe,  $x^{28}$  represents Ser or Ala  $x^{29}$  represents Ser or Cys,  $x^{30}$  represents Ile or Leu  $x^{31}$  represents Met or Lys,  $x^{32}$  represents Ala, Asn, Asp or Glu and  $x^{34}$  represents His or Pro,  $x^{37}$  represents Gly or Ala,  $x^{38}$  represents Phe or Leu,  $x^{43a}$  represents a single bond or Asp,  $x^{44}$  represents Gly or Ala,  $x^{50}$  represents Ala or Thr,  $x^{51}$  represents Glu, Pro or Glu  $x^{52}$  represents Ala or Thr, and  $x^{54}$  represents Ser or Pro,  $x^{59}$  represents Leu or Met,  $x^{61}$  represents Glu or Ala,  $x^{63}$  represents Ile or Thr,  $x^{64}$  represents Phe or Ala,  $x^{67}$  represents Phe or Ala,  $x^{68}$  represents Thr or Ser,  $x^{70}$  represents Lys or Glu,  $x^{77}$  represents Asp or Glu,  $x^{78}$  represents Glu or Gln,  $x^{79}$  represents Asp, Thr or Ser,  $x^{82}$  represents Asp or Glu  $x^{85}$  represents Cys, Tyr or Ser,  $x^{94}$  represents Asp or Asn,  $x^{98}$  represents Cys or Leu,  $x^{99}$  represents Val or Thr,  $x^{100}$  represents Met, Ile or Asn, either  $x^{101}$  represents Gln, Tyr or Phe,  $x^{102}$  represents Glu, Gly, Ser or Ala,  $x^{103}$  represents Glu, Val or Lys,  $x^{104}$  represents Arg, Gly, Thr, Leu or Ala,  $x^{105}$  represents Val, Asp, Met or Thr,  $x^{106}$  represents Gly, Thr, Glu, Leu or Asn,  $x^{107}$  represents Glu, Asn or Tyr,  $x^{108}$  represents Thr or Val,  $x^{109}$  represents Pro or Gln,  $x^{110}$  represents Leu or Arg and  $x^{111}$  represents Met, Lys or Leu,  
or  $-x^{101}-x^{102}-x^{103}-x^{104}-x^{105}-x^{106}-x^{107}-$   
 $x^{108}-x^{109}-x^{110}-x^{111}-$  represents Met, Lys or Leu,  
 $-x^{112}$  represents Asn, Lys or Ala,  $x^{113}$  represents Ala, Glu or Ile,  $x^{114}$  represents Asp or His,  $x^{115}$  represents Ser or Phe,  $x^{120}$  represents Lys or Arg,  $x^{124}$  represents Arg or Gln,  $x^{131}$  represents Thr or Lys,  $x^{137}$  represents Pro or Ala,  $x^{138}$  represents Cys or Ser,

5  $x^{148}$  represents Met or Leu,  $x^{151}$  represents Leu or Phe  
 and  $x^{153}$  represents Leu or Phe, the amino acids being  
 selected such that an amino acid is present in at least  
 one position selected from  $\alpha_2$  positions 12-16, 21,  
 28, 29, 30, 32, 37, 44, 61, 64, 67, 98-115, 137, 138 and  
 148 which differs from the amino acid in the  
 corresponding position of IFN- $\alpha_2$ , IFN- $\alpha_1$  and  
 IFN- $\alpha$  and/or selected such that an amino acid is  
 absent from at least one position selected from  $\alpha_2$   
 10 positions 101-110;

or the amino acids being selected such that  
 an amino acid is present in at least one position  
 selected from  $\alpha_2$  positions 27, 31, 59, 151, 160 and  
 163 which is the same as the amino acid in the  
 15 corresponding position of IFN- $\alpha_1$ , the amino acids at  
 $\alpha_2$  positions other than positions 1 to 11, 27,  
 31, 43a, 59, 100, 102-104, 106, 112, 113, 151 and 156 to  
 165 being the same as the amino acids in the  
 corresponding positions of IFN- $\alpha_2$  or a  $N_{1-11}$  and/or  
 20  $C_{1-11}$  truncated analogue thereof and the  $N_{3-11}$  truncated  
 analogues of IFN- $\alpha_2$ , which may if desired be  $C_{1-11}$   
 truncated, with the proviso that a  $N_3$ -truncated analogue  
 of IFN- $\alpha_2$  is also  $C_{7-11}$  truncated.

2. A polypeptide as claimed in claim 1 wherein  $x^1$   
 25 represents Cys,  $x^2$  represents Asp or Trp,  $x^3$  represents  
 Leu or Cys,  $x^4$  represents Pro or Gln,  $x^5$  represents Glu,  
 Gln or Asp,  $x^6$  represents Thr or Pro,  $x^7$  represents His  
 or Tyr,  $x^8$  represents Ser,  $x^9$  represents Leu,  $x^{10}$   
 30 represents Asp, Gly or Ala,  $x^{11}$  represents Asn, Ser or  
 Ala,  $x^{155}$  represents Thr,  $x^{155}$  represents Thr,  $x^{156}$   
 represents Asn,  $x^{157}$  represents Leu,  $x^{158}$  represents  
 Gln,  $x^{159}$  represents Glu or Lys,  $x^{160}$  represents Arg,  
 Ser or Ile,  $x^{161}$  represents Leu,  $x^{162}$  represents Arg,  
 35  $x^{163}$  represents Arg or Ser,  $x^{164}$  represents Lys and  $x^{165}$   
 represents Glu or Asp.

3. A polypeptide as claimed in claim 1 which is  
 $N_{1-3}$  and/or  $C_{1-10}$  truncated.

4. A polypeptide as claimed in claim 1 of the formula II:-

Cys-Asp-Leu-Pro-X<sup>5a</sup>-Thr-His-Ser-Leu-X<sup>10a</sup>-X<sup>11a</sup>-Arg-Arg-

5 Thr-Leu-Met-Leu-Leu-Ala-Gln-Met-Arg-Lys-Ile-Ser-Leu-

X<sup>27a</sup>-Ser-Cys-Leu-X<sup>31a</sup>-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-

10 Gln-Glu-Glu-Phe-X<sup>43a</sup>-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-

Tyr-Ile-Pro-Val-Leu-His-Glu-X<sup>59a</sup>-Ile-Gln-Gln-Ile-Phe-

Asn-Leu-Phe-Ser-Thr-Lys-Asp-Ser-Ser-Ala-Ala-Trp-Asp-

15 Glu-Thr-Leu-Leu-Asp-Lys-Phe-Tyr-Thr-Glu-Leu-Tyr-Gln-

Gln-Leu-Asn-Asp-Leu-Glu-Ala-Cys-Val-X<sup>100a</sup>-Gln-

X<sup>102a</sup>-X<sup>103a</sup>-X<sup>104a</sup>-Val-X<sup>106a</sup>-Glu-Thr-Pro-Leu-Met-

20 X<sup>112a</sup>-X<sup>113a</sup>-Asp-Ser-Ile-Leu-Ala-Val-Arg-Lys-Tyr-Phe-

Gln-Arg-Ile-Thr-Leu-Tyr-Leu-Lys-Glu-Lys-Lys-Tyr-Ser-

25 Pro-Cys-Ala-Trp-Glu-Val-Val-Arg-Ala-Gln-Ile-Met-Arg-

Ser-X<sup>151a</sup>-Ser-Leu-Ser-Thr-Asn-Leu-Gln-Glu-X<sup>160a</sup>-

30 Leu-Arg-X<sup>163a</sup>-Lys-Glu (in which X<sup>5a</sup> represents Glu or Gln, X<sup>10a</sup> represents Asp or Gly, X<sup>11a</sup> represents Asn or Ser, X<sup>27a</sup> represents Phe or Ser, X<sup>31a</sup> represents Lys or Met, X<sup>43a</sup> represents Asp or a single bond, X<sup>59a</sup> represents Met or Leu, X<sup>100a</sup> represents Met or Ile, X<sup>102a</sup> represents Glu or Gly, X<sup>103a</sup> represents Glu or Val, X<sup>104a</sup> represents Arg or Gly, X<sup>106a</sup> represents Gly or Thr, X<sup>112a</sup> represents Asn or Lys, X<sup>113a</sup> represents Ala or Glu, X<sup>151a</sup> represents Leu or Phe, X<sup>160a</sup> represents Arg or Ser and X<sup>163a</sup> represents Arg or Ser

with the proviso that the amino acids are selected such that at least one of x<sup>27a</sup>, x<sup>31a</sup>, x<sup>59a</sup>, x<sup>151a</sup>, x<sup>160a</sup> and x<sup>163a</sup>, represents the same amino acid as that in the corresponding position of IFN- $\alpha_1$ ).

5        5.        A polypeptide as claimed in claim 1 of the formula 11 as defined in claim 4 in which  
          x<sup>43a</sup> represents Asp, x<sup>100a</sup> represents Ile, x<sup>102a</sup>  
          represents Gly, x<sup>103a</sup> represents Val, x<sup>104a</sup> represents  
          Gly, x<sup>106a</sup> represents Thr, x<sup>112a</sup> represents Lys and  
          x<sup>113a</sup> represents Glu with the proviso that at least one  
 10       of x<sup>5a</sup>, x<sup>10a</sup>, x<sup>11a</sup>, x<sup>151a</sup>, x<sup>160a</sup> and x<sup>163a</sup> represents an  
          amino acid which differs from the amino acid in the  
          corresponding position of IFN- $\alpha_2$ .

         6.        A polypeptide as claimed in claim 1 wherein x<sup>1</sup>  
          represents Cys, x<sup>2</sup> represents Asp or Trp, x<sup>3</sup> represents  
 15       Leu or Cys, x<sup>4</sup> represents Pro or Glu, x<sup>5</sup> represents Gln  
          or Asp, x<sup>6</sup> represents Thr or Pro, x<sup>7</sup> represents His or  
          Tyr, x<sup>8</sup> represents Ser, x<sup>9</sup> represents Leu, x<sup>10</sup>  
          represents Gly or Ala, x<sup>11</sup> represents Ser or Ala, x<sup>16</sup>  
          represents Leu or Met, x<sup>22</sup> represents Arg, x<sup>23</sup>  
 20       represents Lys, x<sup>26</sup> represents Leu, x<sup>27</sup> represents Phe,  
          x<sup>31</sup> represents Lys, x<sup>34</sup> represents His, x<sup>38</sup> represents  
          Phe, x<sup>43a</sup> represents a single bond, x<sup>50</sup> represents Ala,  
          x<sup>51</sup> represents Glu, x<sup>52</sup> represents Thr, x<sup>54</sup> represents  
          Pro, x<sup>63</sup> represents Ile, x<sup>68</sup> represents Ser, x<sup>70</sup>  
 25       represents Lys, x<sup>77</sup> represents Asp, x<sup>78</sup> represents Glu,  
          x<sup>79</sup> represents Thr, x<sup>82</sup> represents Asp, x<sup>85</sup> represents  
          Tyr, x<sup>94</sup> represents Asp, x<sup>105</sup> represents Val, Asp or  
          Thr, x<sup>106</sup> represents Thr, Leu or Asn, x<sup>120</sup>  
          represents Arg, x<sup>124</sup> represents Gln, x<sup>131</sup> represents  
 30       Lys, x<sup>151</sup> represents Phe, x<sup>153</sup> represents Leu, x<sup>156</sup>  
          represents Asn, x<sup>157</sup> represents Leu, x<sup>158</sup> represents  
          Gln, x<sup>159</sup> represents Glu, x<sup>160</sup> represents Ser, x<sup>161</sup>

represents Leu,  $x^{162}$  represents Arg,  $x^{163}$  represents Ser,  $x^{164}$  represents Lys and  $x^{165}$  represents Glu.

7. A polypeptide as claimed in claim 1 of the formula:-

5 Cys  $x^2x^3x^4x^5x^6x^7$ -Ser-Leu- $x^{10c}$ -Ser-Arg-Arg-Thr-Leu-Met-  
 [Leu-Leu-Ala-Gln-Met-Arg-Lys-Ile-Ser-Leu-Phe-Ser-Cys]  
 [Leu-Lys-Asp-Arg-His-Asp-Phe- $x^{37}$ -Phe-Pro-Gln-Glu-Glu]  
 10 [Phe- $x^{44}$ -Asn-Gln-Phe-Gln-Phe-Gln-Lys-Ala-Glu-Thr]  
 [Ile-Pro-Val-Leu-His-Glu- $x^{59}$ -Ile-Gln-Gln-Ile-Phe-Asn]  
 [Leu-Phe-Ser-Thr- Lys-Asp-Ser-Ser-Ala-Ala-Trp-Asp-Glu]  
 15 [Thr-Leu-Leu-Asp-Lys-Phe-Tyr-Thr-Glu-Leu-Tyr-Gln-Gln]  
 [Leu-Asn- Asp-Leu-Glu-Ala- $x^{98c}$ - $x^{99c}$ - $x^{100c}$ - $x^{101c}$   
 20 [ $x^{102c}$ - $x^{103c}$ - $x^{104c}$ - $x^{105c}$ - $x^{106c}$ - $x^{107c}$ - $x^{108}$ - $x^{109}$ - $x^{110}$   
 [ $x^{111c}$ - $x^{112c}$ - $x^{113c}$ - $x^{114}$ -Ser-Ile-Leu-Ala-Val-Arg-Lys]  
 [Tyr-Phe -Gln-Arg-Ile-Thr-Leu-Tyr-Leu-Lys-Glu-Lys-Lys]  
 25 [Tyr-Ser-Pro-Cys-Ala-Trp-Glu-Val-Val-Arg-Ala-Glu-Ile]  
 [Met- Arg-Ser-Phe-Ser-Leu-Ser-Thr-Asn-Leu-Gln-Glu-Ser]  
 30 [Leu-Arg-Ser-Lys-Glu

(wherein  $x^2$  represents Asp or Trp,  $x^3$  represents Leu or Cys,  $x^4$  represents Pro or Gln,  $x^5$  represents Gln or Asp,  $x^6$  represents Thr or Pro,  $x^7$  represents His or Tyr,  $x^{10c}$  represents Gly or Ala,  $x^{37}$  represents Gly or Ala,  $x^{44}$  represents Gly or Ala,  $x^{59}$  represents Met or Leu,

x<sup>98c</sup> represents Cys or Leu, x<sup>99c</sup> represents Val or Thr, x<sup>100c</sup> represents Ile, Met or Asn, x<sup>101c</sup> represents Gln or Tyr, x<sup>102c</sup> represents Gly, Ala or Ser, x<sup>103c</sup> represents Val, x<sup>104c</sup> represents Gly, Ala or Thr, x<sup>105c</sup> represents Val or Asp, x<sup>106c</sup> represents Thr or Leu, x<sup>107c</sup> represents Glu or Asn, x<sup>108</sup> represents Thr or Val, x<sup>109</sup> represents Pro or Gln, x<sup>110</sup> represents Leu or Arg, x<sup>111c</sup> represents Met or Lys, x<sup>112c</sup> represents Lys or Ala, x<sup>113c</sup> represents Glu or Ile, and x<sup>114</sup> represents Asp or His) and corresponding polypeptides in which Cys at positions 29 and 138 is replaced by Ser, the amino acids being selected such that at least in one of positions 2-7,10,37,44,59 and 99-114 an amino acid is present which differs from the amino acid in the corresponding position of IFN- $\alpha_2$ .

8. A polypeptide as claimed in claim 1 in which -x<sup>98</sup>-x<sup>99</sup>-x<sup>100</sup>-x<sup>101</sup>-x<sup>102</sup>-x<sup>103</sup>-x<sup>104</sup>-x<sup>105</sup>-x<sup>106</sup>-x<sup>107</sup>-x<sup>108</sup>-x<sup>109</sup>-x<sup>110</sup>-x<sup>111</sup>-x<sup>112</sup>-x<sup>113</sup>-x<sup>114</sup>- represents the moiety -Leu-Thr-Asn-Tyr-Ser-Val-Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Ala-Ile-His-.

9. A polypeptide as claimed in claim 1 in which at least one of x<sup>10</sup>, x<sup>37</sup>, x<sup>44</sup>, x<sup>102</sup> and x<sup>104</sup> represents alanine.

10. A polypeptide as claimed in claim 1 in which x<sup>59</sup> represents leucine.

11. A polypeptide as claimed in claim 1 selected from the group consisting of:-

IFN- $\alpha_2$ (4-155)  
 endo-Asp<sup>43a</sup>-[Leu<sup>151</sup>,Arg<sup>160,163</sup>]IFN- $\alpha_2$   
 [gamma(98-114)<sup>98-114</sup>]IFN- $\alpha_2$   
 [Met<sup>100</sup>, gamma(101-114)<sup>101-114</sup>]IFN- $\alpha_2$   
 [gamma(2-7), gamma(98-114)]IFN- $\alpha_2$   
 [Leu<sup>59</sup>]IFN- $\alpha_2$   
 [Ala<sup>10,37,44,102,104</sup>]IFN- $\alpha_2$   
 [Ser<sup>29,138</sup>]IFN- $\alpha_2$

12. A process for producing a polypeptide as defined in claim 1 which comprises culturing a microorganism, the microorganism having been transformed with a replicable plasmidic expression vehicle comprising genetic material coding for the said polypeptide whereby to effect expression of the said polypeptide and recovering the said polypeptide thereby expressed.

13. A transformant microorganism capable of expressing a polypeptide as defined in claim 1, the said microorganism comprising a replicable plasmidic expression vehicle, which vehicle comprises genetic material coding for the said polypeptide.

14. A process for the preparation of a transformant microorganism as defined in claim 13 which comprises transforming a microorganism by the insertion therein of a replicable plasmidic expression vehicle, which vehicle comprises genetic material coding for a polypeptide as defined in claim 1.

15. A replicable plasmidic expression vehicle capable, in a transformant microorganism, of expressing a polypeptide as defined in claim 1.

16. A process for the preparation of a replicable plasmidic expression vehicle as defined in claim 15 which comprises inserting a gene coding for a polypeptide as defined in claim 1 into a vector therefor at an appropriate insertion site.

17. A process for the preparation of a replicable plasmidic expression vehicle as defined in claim 15 which comprises inserting a nucleotide sequence coding for a portion of the polypeptide as defined in claim 1 into a vector comprising a promoter sequence and a nucleotide sequence(s) coding for the remainder of the polypeptide as defined in claim 1 at an appropriate insertion site whereby a replicable plasmidic expression vehicle is obtained capable of directing the synthesis

of the said polypeptide in a transformant micro-organism.

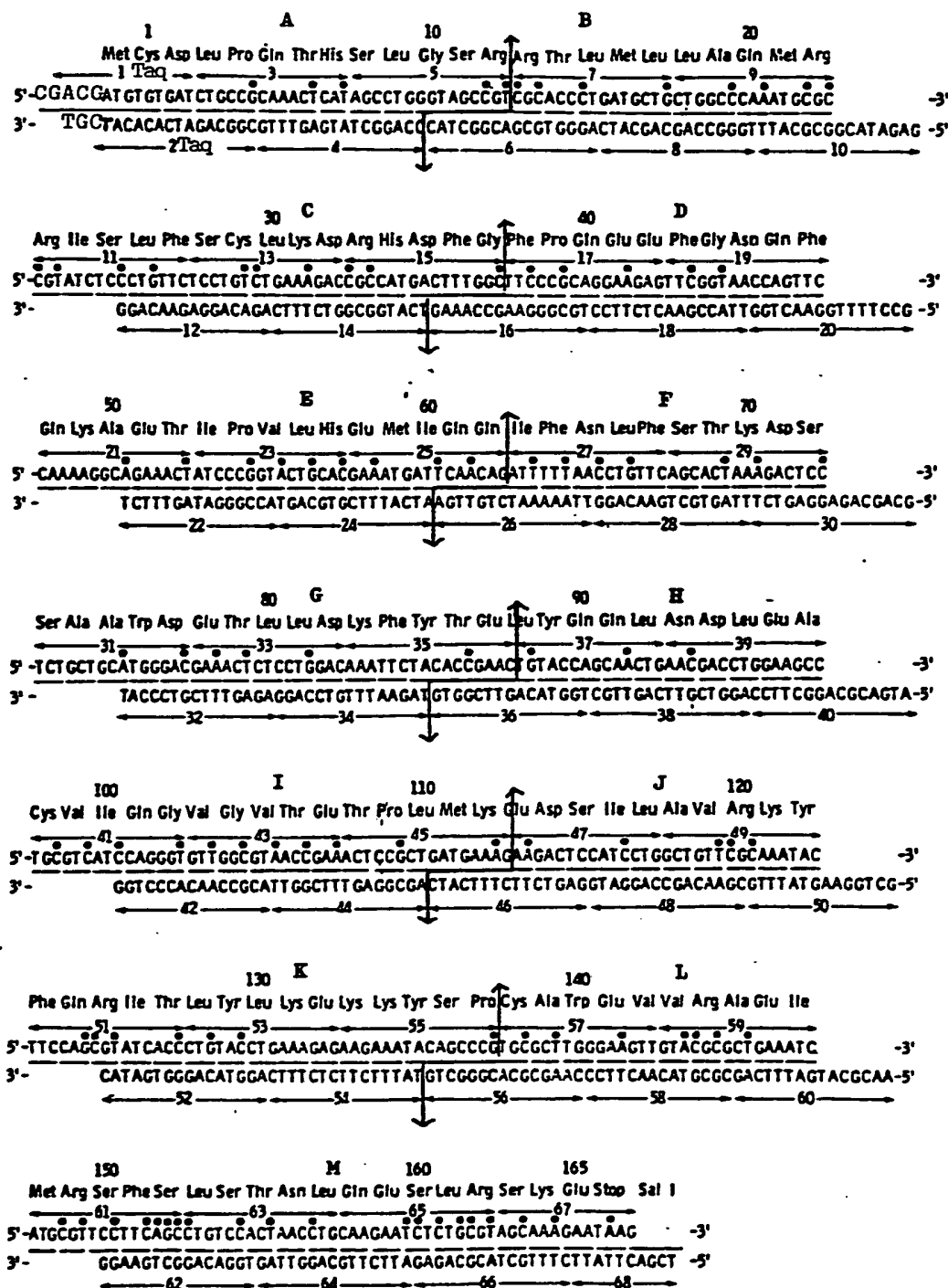
18. A DNA sequence that encodes for a polypeptide as defined in claim 1.

5 19. A portable expression unit comprising the EcoRI-SalI segment of the plasmid pSTPl in which a DNA sequence as defined in claim 18 is present between the ClaI and SalI restriction sites of the said segment.

10 20. A pharmaceutical composition comprising as active ingredient at least one polypeptide as defined in claim 1 in association with a pharmaceutically acceptable carrier or excipient.

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Figure 1 :



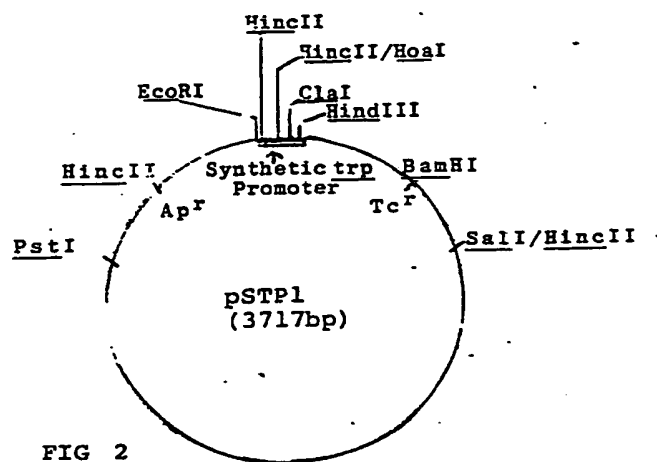


FIG 2

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# EUROPEAN SEARCH REPORT

Application number

EP 86 30 0304

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	WO-A-8 304 053 (APPLIED MOLECULAR GENETICS) * Claims, especially 38; figures *	1,2,4- 8,12- 18	C 07 K 15/26 C 12 N 15/00 C 12 P 21/02 A 61 K 45/02
X	SCIENCE, vol. 212, 5th June 1981, pages 1159-1162, AAAS; R. LAWN et al.: "DNA sequence of two closely linked human leukocyte interferon genes" * Page 1161, example lambda 2C1 *	1,2,4- 8,12- 18	
Y	WO-A-8 400 776 (CETUS CORP.) * Claims *	1	
Y	EP-A-0 100 561 (CIBA-GEIGY) * Claims; figures 10-14 *	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Y	EP-A-0 089 692 (BRISTOL-MEYERS CO.) * Claims *	1	C 12 N C 12 P
Y	WO-A-8 302 460 (CETUS CORP.) * Claims *	1	
Y	WO-A-8 302 459 (CETUS) * Claims *	1	
	--- -/-		
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 13-06-1986	Examiner DELANGHE L.L.M.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			

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Y	WO-A-8 302 458 (CETUS) * Claims *	1	
Y	WO-A-8 302 457 (CETUS) * Claims *	1	
Y	EP-A-O 076 489 (CIBA-GEIGY) * Claims *	1	
Y	EP-A-O 072 541 (HOFFMANN-LA ROCHE) * Claims; figures *	1	
Y	EP-A-O 062 971 (ICI) * Claims; figures *	1	
Y	EP-A-O 051 873 (GENENTECH) * Claims; figures *	1	
Y	EP-A-O 032 134 (BROGEN) * Claims 21-24 *	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 13-06-1986	Examiner DELANGHE L. L. M.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone  Y : particularly relevant if combined with another document of the same category  A : technological background  O : non-written disclosure  P : intermediate document</p> <p>T : theory or principle underlying the invention  E : earlier patent document, but published on, or after the filing date  D : document cited in the application  L : document cited for other reasons  &amp; : member of the same patent family, corresponding document</p>			